

## REMARKS

Claims 1-6, 8-14, 16, and 30-39 are now pending in the application. Claims 1-6, 8-14, 16, and 30-39 are rejected and claims 3 and 11 are objected to. Claims 1, 3, 8, 10, 11, 12, 13, 14, 16, 30, 32, 37, and 39 have been amended. Claims 4, 35, 36, and 38 have been cancelled. New claims 40 and 41 have been added.

Applicants would like to acknowledge the grant of the benefit of the filing dates of: provisional application serial number 60/033,193, filed December 18, 1996; and non-provisional applications 08/989,332, filed December 11, 1997, and 09/422,073, filed October 21, 1999. Applicant would like to point out that the subject application does not claim priority to international application PCT/US97/23014, filed December 12, 1997. Accordingly, a Request for Corrected Filing Receipt will be filed shortly with the U.S. Patent and Trademark Office removing the claim of priority to PCT/US97/23014.

Applicant would like to point out for the record that the transitional term "comprising" which is used in several of the claims is synonymous with the transitional term "containing" also used in several of the claims. Both terms are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. See, e.g. *Genentech, Inc. v. Chiron Corp.*, 112 F.3d 495, 501, 42 USPQ2d 1608, 1613 (Fed. Circuit 1997) and M.P.E.P. §2111.03.

### **The Amendments**

The first paragraph of the specification has been amended to update the status of a priority document. Also, the specification has been amended at page 6, line 18, and page 20, line 19 to correct clerical errors. In addition, the specification has been amended at page 14, line 18, page 25, line 18, and page 26, line 22, to provide the patent numbers of applications that have issued since the filing of the subject application.

The specification has been amended to correct a number of minor typographical and clerical errors. In particular, the spelling of "pantetheinylation" and related words has been corrected at various locations throughout the specification.

In addition, Example 8 of the specification has been amended to change the tense from the past to the present throughout the Example to reflect that the activities described have not actually been performed. At the time the application was prepared, the inventors believed that the activities described in Example 8 had been performed. However, the inventors later learned that the linker described in the Example had not been used. The linker described in the Example had been ordered from a commercial supplier, but the supplier provided a linker in which the sequence "AAA GCC" was changed to "AAT CC". This change created a stop codon in the 6-MSAS coding sequence so that a fusion protein is not produced. Accordingly, the Applicants have amended the Example to employ the present tense throughout.

Claim 1 has been amended for clarification and to provide proper antecedent support. Support can be found, for example, at page 3, line 25 to page 4, line 5, at page 6, lines 13-14, and page 7, lines 21-23. Claim 3 has been amended to provide antecedent basis. Support can be found, for example, at page 3, lines 20-21. Claim 8 has been amended to provide antecedent basis and for clarification. Support can be found, for example, at page 3, lines 11-17, and with originally filed claim 1. Claim 10 has been amended to clarify the claimed invention. Support can be found, for example, at page 12, line 17 to page 13, line 21. Claim 11 has been amended for consistency of claim language. Claim 12 has been amended to provide antecedent basis and for clarification. Support can be found, for example, at page 3, lines 11-17, and line 25 to page 4, line 5. Claim 13 has been amended to more clearly define the invention. Claim 14 has been amended for clarification. Support can be found, for example, at page 2, lines 21-24, page 4, lines 10-16, page 6, lines 15 and 24, page 7, line 6, and page 14, lines 11-22. Claims 16, 32, and 37 have been amended for clarification. Support can be found, for example, at page 7, lines 21-23. Claim 30 was amended for clarification. Claim 37 has been amended to clarify the invention and to incorporate the limitations of claim 35 in which it depends from. Support can be found, for example, at page 2, line 28 to page 3, line 2, and in originally filed claim 35. Claim 39 has been amended for clarification. Support can be found, for example, at page 1, lines 9-11. Claim 40 has been added. Support can be found, for example, at page 8, lines 6-15. Claim 41

has been added. Support can be found, for example, at page 16, lines 21-25, page 18, lines 9-10, and pages 21-22.

No new matter has been added.

### **Objections to the Specification**

The specification is objected to for lacking complete continuity data in the first paragraph. Applicant directs the Examiner's attention to a Preliminary Amendment mailed July 11, 2002, in which the complete continuity data for the subject application was provided. A copy of the Preliminary Amendment is enclosed. In addition, in the current Amendment, Applicant has corrected the status of the provisional application as "abandoned."

The specification is objected to for not updating references to patent application numbers throughout the specification. Applicant thanks Examiner for pointing out this oversight. The specification has been amended accordingly.

The specification is also objected to for containing several confusing terms and for containing typographical errors. Accordingly, the specification has been amended to correct these errors.

### **Objections to the Claims**

Claim 3 is objected to for lacking antecedent basis and claim 11 is objected to for lacking consistency. Appropriate amendments have been made to the claims.

### **Rejection of Claims Under 35 U.S.C. § 112, First Paragraph**

Claims 1-6, 16, 32, 34, 37, and 39 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention.

The Examiner asserts that the instant claims are directed to host cells containing expression systems for polyketide synthases (PKSs) and holo acyl carrier protein (ACP) synthases where the claimed product is defined by its functional characteristics with respect to the ACP synthase component. The Examiner also states that a holo ACP synthase is not defined in the instant specification or in the art, other than by its ability to catalyze phosphopantetheinyl transfer to activate ACPs of PKSs. Applicant respectfully disagrees with the Examiner's remarks for the reasons set forth below.

*University of California v. Eli Lilly and Co.*, 1997, U.S. App. LEXIS 18221, cited by the Examiner, quotes *Fiers v. Revel*, 984 F.2d 1164, 1171, 25 USPQ2d 1601, 1606 (Fed Cir. 1993) as stating that "An adequate written description of a DNA ... requires a precise definition, such as by structure, formula, chemical name, or physical properties..." (emphasis added). Accordingly, "an adequate written description of a DNA requires more than a mere statement that it is part of the invention ... what is required is a description of the DNA itself." *Id.* at 1170, 25 USPQ2d at 1606.

Respectfully, it is believed that the facts in the present case do not reflect those in the cited case. Specifically, the claims in the *Lilly* case were directed to cDNA's-*i.e.*, the precise reverse transcripts of mRNA encoding proinsulin from various species. Therefore, the invention in *Lilly* was the DNA and not as with the claims at issue, the use of DNA (art known holo ACP synthases).

Nonetheless, Applicant believes that an adequate written description of a holo ACP synthase is provided in the specification. A "precise definition" of holo ACP synthases, "such as by ... physical properties", is provided, for example, at page 7, line 21 to page 8, line 15, and pages 18, 19, and 20.

In addition, MPEP §2163, states that "Whether the specification shows that applicant was in possession of the claimed invention is ... a factual determination reached by considering a number of factors. Factors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, partial structure, physical and/or

chemical properties, functional characteristics *alone or* coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention. Disclosure of *any* combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is *sufficient*. See *University of California v. Eli Lilly and Co.*, 119 F.3d. at 1568, 43 USPQ2d at 1406. Patents and printed publications in the art should be relied upon to determine whether an art is mature and what the level of knowledge and skill is in the art. In most technologies which are mature, and wherein the knowledge and level of skill in the art is high, a written description question should not be raised for original claims even if the specification discloses only a method of making the invention and the function of the invention. See, e.g., *In Re Hayes Microcomputer Products, Inc. Patent Litigation*, 982 F.2d. 1527, 1534-35, 25 USPQ2d 1241, 1246 (Fed. Cir. 1992)” (emphasis added).

Lambalot (see Lambalot, R.H., *et al.*, A new enzyme superfamily - the phosphopantetheinyl transferase, *Chemistry & Biology*, 3:923-936 (November 1996), of which the publication date is earlier than the earliest priority date of the subject application, namely December 18, 1996 (hereinafter “Lambalot”)) identifies a large family of proteins that have been shown to be or are believed to be phosphopantetheinyl transferases including: ACPS, EntD, Sfp, Psf-1, Gsp, Lpa-14, NshC, o195, Het1, SYCCPNC, Lys5, CELT04G9, H10152, FAS2, Bli, 1314154, and CELT04G9. In addition, several of the proteins have been found in several different organisms. A copy of Lambalot is enclosed for the convenience of the Examiner.

Accordingly, a combination of the knowledge of one skilled in the art, wherein the knowledge and level of skill in the art is high, and the description in the specification of the function of holo ACP synthases, would lead one of skill in the art to the conclusion that Applicant was in possession of the claimed invention. In addition, Lambalot establishes that the art is mature and what the level of knowledge and skill is in the art.

The Examiner also states on page 21-22, that *E. coli* fatty acid synthase holo ACP synthase (ACPS) does not function effectively, and that the difference between effective holo

ACP synthases and those that are not effective at pantotheinylation of PKS systems is not structurally defined. Accordingly, claims 1, 16, 32, and 37 have been amended to include the phrase “a holo ACP synthase capable of being expressed and effective in the pantotheinylation of said PKS.”

In conclusion, Applicant respectfully requests that the rejection under 35 U.S.C. § 112, first paragraph, written description be withdrawn.

Claims 1-6, 16, 32, 34, 37, and 39 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention.

The Examiner asserts that the specification, while being enabling for host cells containing expression systems for polyketide synthases (PKSs) and *particular* expression systems for holo acyl carrier protein (ACP) synthases, does not reasonably provide enablement for host cells containing expression systems for PKSs and *all* expression systems for holo ACP synthases.

The Examiner further asserts that no guidance is presented for the purpose of determining expression systems for holo ACP synthases, i.e. determining genes encoding holo ACP synthases, with no discussion of homology among the holo ACP synthases used.

In addition, the Examiner states that though applicants have enabled the use of expression systems using holo ACP synthases EntD, GsP, and Sfp to produce holo ACPs of type I PKSs in *E. coli* host cells, Applicant has not enabled the use of these holo ACPs with type II PKSs.

Applicant respectfully disagrees and will discuss below why the specification does enable a person of skill in the art to make and/or use the invention commensurate in scope with the claims without undue experimentation. Applicant will discuss factual considerations as set forth *In re Wands* 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988), and cited by the Examiner.

Starting at page 7, line 21, *et seq.*, of the specification, guidance is provided instructing one skilled in the art to determine whether an expression system requires the presence of a holo ACP synthase in order to obtain polyketide production. The specification describes the

importance of the holo ACP synthase in effecting pantetheinylation of the acyl carrier protein. If a host cell lacks a phosphopantetheinylating enzyme that behaves as a holo ACP synthase, the specification provides a description of the means for conferring this activity by supplying an expression system for this enzyme. The specification then provides direction as to which holo ACP synthase to choose. Specifically, “holo ACP synthases associated with fatty acid synthesis are not suitable; rather, synthases associated specifically with polyketide synthesis or with synthesis of nonribosomal proteins are useful” in the invention. Further instruction is provided regarding which holo ACP synthase to choose, “the modular and fungal PKS systems are not activated by phosphopantetheinylation effected by the phosphopantetheinylation enzymes indigenous to *E. coli*; however, enzymes derived from *Bacillus*, in particular the gramicidin holo ACP synthase of *Bacillus brevis* and the surfactin-related holo-ACP synthase from *Bacillus subtilis* can utilize the modular and fungal PKS ACP domains as substrates.”

One of skill in the art would understand how to choose a holo ACP synthase and how to use it in the expression systems of the current invention. At the time of the invention, it was known that “all polyketide synthases, fatty acid synthases, and non-ribosomal peptide synthetases require posttranslational modification of their constituent acyl carrier protein domain(s) to become catalytically active.” It was also known that the “inactive apoproteins are converted to their active holo-forms by posttranslational transfer of the 4'-phosphopantetheinyl (P-pant) moiety of coenzyme A to the sidechain hydroxyl of a conserved serine residue in each acyl carrier protein domain.” See *Lambalot id.* Furthermore, Lambalot identifies a large family of proteins that have been shown to be or are believed to be phosphopantetheinyl transferases including: ACPS, EntD, Sfp, Psf-1, Gsp, Lpa-14, NshC, o195, Het1, SYCCPNC, Lys5, CELT04G9, H10152, FAS2, Bli, 1314154, and CELT04G9. In addition, several of the proteins have been found in several different organisms. Additional guidance as to how to manipulate a holo ACP synthase is provided in the specification by working examples 1, 3, 4, 6, and 7.

Therefore, the relatively high level of one skilled in the art makes the amount of experimentation necessary and the amount of direction or guidance needed, beyond what is

provided in the specification, to carry out the claimed invention, minimal and undue. Also, given the state of the prior art, as exemplified in Lambalot, the likelihood of success for one skilled in the art to manipulate the components of holo ACP synthases in the expression systems of the claimed invention is very high.

In addition to type II PKSs being enabled by the specification, the use of type I and fungal PKSs are also enabled by the specification. The general features of aromatic, modular, and fungal PKS systems are shown in Figures 1, 2, and 3, respectively, and described, for example, in the specification at pages 6 and 7. On page 7, starting at line 17, PCT application WO 95/08548, incorporated by reference, describes the construction of hybrid aromatic (type II) PKS systems wherein open reading frames of actinorhodin are included in expression vectors with open reading frames from alternative aromatic systems. Example 11 also describes the use of type II PKSs in the host cells, vectors, and methods of the invention. Also, working examples 1, 2, 4, and 7, provide guidance to one skilled in the art as to how to manipulate components of fungal PKSs.

Furthermore, the Examiner has herself stated that “copious amounts of structural ... data on PKSs, wherein the name ‘polyketide synthase’ dictates a particular structure” are present in the art. Therefore, the relatively high level of one skilled in the art makes the amount of experimentation necessary and the amount of direction or guidance needed, beyond what is provided in the specification, to carry out the claimed invention, minimal and undue. Also, given the state of the prior art, as explained by the Examiner, the likelihood of success for one skilled in the art to manipulate the components of type I, type II, and fungal PKS in the expression systems of the claimed invention is very high.

In addition, the experiments described in Example 6 provide guidance to one skilled in the art to determine if a new holo ACP synthase is suitable for the expression system of the invention. Therefore, Applicant has enabled the identification of new holo ACP synthases for use in the invention.



The attention of the Office is called to the decision in *Ex parte Mark*, 12 USPQ2d 1904 (Bd. Pat. App. & Int. 1989) which is quite similar to the facts here. In that case, claims were drawn to muteins where at least one non-essential cysteine was replaced by another amino acid in any protein at all. The Examiner had rejected the claims on the basis that only three proteins were illustrated and the choice of the appropriate cysteine in other proteins was not taught. The Board held that the claims were fully supported as it was well within ordinary skill to test the limited number of possible muteins for retention of activity based on the amino acid sequence of any arbitrary protein. A copy of this decision is enclosed. Similarly, here, it is well within ordinary skill to screen for a holo ACP synthase, capable of being expressed and effective in the pantotheinylation of a PKS. Accordingly, it is believed that the claims as presented are of appropriate scope.

For the reasons presented above, Applicant believes that the basis of the rejection under 35 U.S.C. § 112, first paragraph, scope of enablement, fails to rise to the level of a *prima facie* case and should be withdrawn.

Claim 4 stands rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention.

The Examiner asserts that the specification, while being enabling for cells containing expression systems for fusion proteins comprising a fungal PKS and a holo ACP synthase, does not reasonably provide enablement for cells containing expression systems for fusion proteins comprising an aromatic or modular PKS and a holo ACP synthase.

Although Applicant disagrees with the Examiner for the reasons set forth below, in an effort to expedite prosecution, Applicant has cancelled claim 4.

The Examiner states that the fungal PKS is the simplest form of a PKS and does not necessarily correlate with aromatic or modular PKS systems, and that the specification provides no working examples or guidance for the production of aromatic or modular PKS fusion protein

systems. Applicant respectfully disagrees with the Examiner's remarks for the reasons set forth below.

First, as described in the specification at page 7, lines 4-8, a fungal PKS, as exemplified by 6-MSAS, "has some similarity to both the aromatic and modular PKS. It has [a] ... KS, AT, dehydratase (DH), KR, and ACP. Thus, it looks similar to a single module of a modular PKS.... Unlike an aromatic PKS, it does not include a CLF...." Therefore, the fungal PKS system *does* correlate, "or have corresponding characteristics" (*see* The American Heritage College Dictionary, 312 (3ed.1993)) with the modular or aromatic PKS systems.

Second, guidance is provided for the production of a fusion protein by Example 8 of the specification. Also, since the Examiner has herself stated that "copious amounts of structural ... data on PKSs, wherein the name 'polyketide synthase' dictates a particular structure" are present in the art, it would not be undue experimentation for one skilled in the art to follow the teachings of Example 8 in order to make a fusion protein comprising a type I or type II PKS and a holo ACP synthase. Furthermore, the molecular biology techniques required to make a fusion protein were well known at the time of the invention. In addition, Applicant respectfully points out to the Examiner that no working examples are required to provide an enabling disclosure.

Therefore, one skilled in the art, armed with the guidance provided by the specification in regards to how to manipulate components of aromatic, modular, or fungal PKSs, and holo ACP synthases, would not have to undergo undue experimentation to obtain cells containing expression systems for fusion proteins comprising an aromatic or modular PKS and a holo ACP synthase.

In conclusion, due to the cancellation of claim 4, the rejection under 35 U.S.C. § 112, first paragraph, scope of enablement, is moot.

### **Rejection of Claims Under 35 U.S.C. § 112, Second Paragraph**

Claim 1 stands rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that the abbreviation “ACP” in line 3 must be defined upon its first appearance in the claims. Applicant has amended claim 1 to define “ACP.”

Accordingly, Applicant asserts that the language of claim 1 is clear and meets the requirements of 35 U.S.C. § 112, second paragraph.

Claim 4 stands rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that antecedent basis for the phrase “the nucleotide sequence” in claim 4 is unclear since claim 1 does not refer to a nucleotide sequence. Applicant has amended claim 1 to provide antecedent support.

Claim 4 has been cancelled, making the rejection under 35 U.S.C. § 112, second paragraph, moot.

Claims 8-16 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claim 15 has been cancelled.

The Examiner states that in claim 8, there is no antecedent basis for the phrases “said first vector” and “said second vector.” Applicant has amended claim 8 to provide antecedent support.

Accordingly, Applicant asserts that the language of claims 8-14 and 16 are clear and meet the requirements of 35 U.S.C. § 112, second paragraph.

Claim 10 stands rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that the phrase “expression system for a cell-based detection system for a functional polyketide” is undefined by the specification and the prior art does not provide a basis to define this phrase. Although Applicant respectfully disagrees, because Applicant believes that the phrase is defined in the specification, for example, at page 12, line 17 to page 13, line 21, and also in the article cited by the Examiner authored by Broach and Thorner, for the purpose of facilitating allowance of the claim, Applicant has amended claim 10 to further clarify the invention.

Accordingly, Applicant asserts that the language of claim 10 is clear and meets the requirements of 35 U.S.C. § 112, second paragraph.

Claims 13-14 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that in claim 13 the antecedent basis for the phrase “said first and second module” is unclear since neither claim 8 nor claim 12 refers to a first or second module. Applicant would like to respectfully point out to the Examiner that the phrase “at least a second module” does appear in claim 12 providing proper partial antecedent support for claim 13. Applicant has amended claim 12 to provide antecedent support by providing the phrase “at least a first module”, and claim 12 has also been amended to clarify the invention.

Accordingly, Applicant asserts that the language of claims 13 and 14 are clear and meet the requirements of 35 U.S.C. § 112, second paragraph.

Claims 13-14 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that in claim 13 the term “different” is unclear due to the copious amounts of mutagenesis and domain swapping found in the art of polyketide synthases. Applicant has amended claim 13 to include the phrase “wherein said first module is that of a first polyketide synthase (PKS) or said second module is that of a second PKS, wherein said first and second PKS are different.”

Accordingly, Applicant asserts that the language of claims 13 and 14 are clear and meet the requirements of 35 U.S.C. § 112, second paragraph.

Claim 14 stands rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that the phrase “said nucleotide sequence encoding at least one module” is unclear because only in claim 12 is a nucleotide sequence mentioned, and then only in reference to the second vector. Applicant has amended claim 12, as described above, to provide antecedent support for claims 13 and 14. In addition, Applicant has amended claim 14 to more clearly define the invention.

Accordingly, Applicant asserts that the language of claim 14 is clear and meets the requirements of 35 U.S.C. § 112, second paragraph.

Claim 14 stands rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that the abbreviations “KR”, “DH”, and “ER” are not defined upon their first occurrence in the claims. Therefore, Applicant has amended claim 14 to more clearly define these terms.

Accordingly, Applicant asserts that the language of claim 14 is clear and meets the requirements of 35 U.S.C. § 112, second paragraph.

Claims 30-39 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that the phrase “functional polyketide synthase catalytic activity” is unclear because polyketide synthases are multifunctional enzymes catalyzing a wide variety of reactions, for example an acyl transferase reaction. In addition, the Examiner inquires whether an antibiotic or polyketide must be produced, and states that to claim a coding region of a catalytic activity is confusing. Applicant believes that the amendments to claim 30 remove the confusion regarding claiming a coding region of a catalytic activity. In addition, claims 35, 36, and 38 have been cancelled. Applicant agrees with the Examiner’s statement that polyketide synthases are multifunctional enzymes catalyzing a wide variety of reactions, for example an acyl transferase reaction, and would like to clarify that the scope of claims 30-39 encompasses a vector that comprises at least one catalytic activity of a polyketide synthase (PKS), to a vector that comprises a group of catalytic activities that make up a PKS that is capable of producing a polyketide or antibiotic.

Accordingly, Applicant asserts that the language of claims 30-34, 37, and 39 are clear and meet the requirements of 35 U.S.C. § 112, second paragraph.

Applicants would like to state that amendments to the claims were not made for reasons of patentability but rather for the purpose of clarifying the invention, see *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co., et al.*, 122 S.Ct. 1831, 1838, 62 USPQ2d 1705, 1710 (2002). The

amendments made to the claims do not narrow the scope of the claims because the substituted phraseology has the same meaning as the previous claim language.

In addition, Applicants respectfully direct the Examiner's attention to the Memorandum dated January 17, 2003, from Deputy Commissioner for Patent Examination Policy Steve G. Kunin, which clarifies the U.S. Patent and Trademark's policy with respect to rejections made under 35 U.S.C. §112, second paragraph.

### **Rejection of Claims Under 35 U.S.C. § 102(e)**

Claims 8, 9, 11, and 12 stand rejected under 35. U.S.C. § 102(e) as being anticipated by U.S. Patent No. 5,712,146 (hereinafter "the '146 patent").

The '146 patent teaches a genus of cells that are capable of expressing a polyketide synthase (PKS) gene cluster and it teaches a genus of vectors that can be used to express the PKS gene cluster. Although the '146 patent does not specifically refer to the subset of cells that are claimed in the subject application, or specifically disclose the use of at least two vectors or at least a vector and a modified chromosome, there is precedent for patentability in such situations. The attention of the Office is called to the decision in *Integra Lifesciences v. Merck*, 331 F.3d 860; 2003 U.S. App. LEXIS 11335; 66 U.S.P.Q.2D (Fed.Cir. 2003) which provides guidance as to the general teachings of in such genus-species relationships. In that case, claims to a cyclic peptide were found to be patentable over a patent that disclosed but did not specifically claim cyclic RGD peptides. A copy of this case is included with the Response.

Claim 8 has been amended to include the phrase "A modified recombinant host cell, which in unmodified form does not produce polyketides, modified to contain either...." As amended, claim 8 discloses a modified recombinant host cell which in unmodified form does not produce polyketides. As described in the specification on page 5, lines 1-8, "hosts such as *E. coli*, yeast, and other microbial systems which do not customarily synthesize polyketides can be made into convenient hosts." In addition, Applicant's invention provides "the opportunity to

produce polyketides in hosts which normally do not produce them, such as *E. coli*, and yeast.”

See specification at page 14, lines 23-24.

Applicant acknowledges the Examiner’s statement that the ‘146 patent does not teach inventions regarding the presence of modified chromosomes.

In conclusion, Applicant asserts that claims 8, 9, 11, and 12 are patentable under 35 U.S.C. § 102(e) over the ‘146 patent.

Claims 30-31 and 33 stand rejected under 35 U.S.C. § 102(e) as being anticipated by the ‘146 patent described above.

The ‘146 patent is cited by the Examiner as teaching a transformation of the pCK7 plasmid containing the *eryAI*, *eryAII*, and *eryAIII* genes and several selectable markers into *Streptomyces coelicolor* CH999 cells to express the erythromycin polyketide synthase (PKS) and, in turn, produce the polyketide product, 6dEB.

The teachings of the ‘146 patent have been discussed *supra*.

In addition, claims 30, 31, and 33 are directed towards a vector adapted for expression in yeast, a yeast cell, and a method of producing a polyketide synthase activity by culturing the yeast cell; *Streptomyces coelicolor* is a bacterium, not a yeast. See Dorland’s Illustrated Medical Dictionary, 1592 (27ed. 1988).

In conclusion, Applicant asserts that claims 30-31 and 33 are patentable under 35 U.S.C. § 102(e) over the ‘146 patent.

### **Rejection of Claims Under 35 U.S.C. § 102(b)**

Claims 30, 31, and 33 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Shen, B., *et al.* (Science, 262:1535-1540 (1993)) (hereinafter “Shen”).

The Examiner asserts that Shen teaches the *S. glaucescens* WMH1077/(pWHM722) transformant that carries the *tcmKLMN* genes under the control of a strong constitutive promoter in ... vector pIJ486 and the culturing of said transformant to express the aromatic, type II



polyketide synthase (PKS) *tcm* KLMN gene products. The Examiner also states that *S. glaucescens* is a yeast host cell.

Present claims 30, 31, and 33 are directed towards a vector adapted for expression in yeast, a yeast cell, and a method of producing a polyketide synthase activity by culturing the yeast cell; *Streptomyces glaucescens* is a bacterium, not a yeast. See Dorland's Illustrated Medical Dictionary, 1592 (27ed. 1988).

Therefore, Applicant asserts that claims 30, 31, and 33 are patentable under 35 U.S.C. § 102(b) over Shen.

Claims 35, 36, and 38 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Gramajo, H.C., *et al.* (J. of Bacteriology, 173:6475-6483 (1991)) (hereinafter "Gramajo").

The Examiner asserts that Gramajo teaches the pT7-7 vector which comprises an ampicillin resistance selectable marker, the T7 gene 10 promoter, and ORFs 1, 2, and 3 of the aromatic, type II polyketide synthase (PKS) gene producing tetracenomycin which ORFs code for ketosynthase and acyl carrier protein activities. In addition, the Examiner states that Gramajo teaches the transformation of the pT7-7 vector into *E. coli* host cells.

While not necessarily agreeing the the Examiner's position, in order to expedite prosecution, claims 35, 36, and 38 have been cancelled, making the rejection moot.

Claims 35, 36, and 38 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Roberts, G.A., *et al.* (Eur. J. Biochem. 214:305-311 (1993)) (hereinafter "Roberts").

Roberts is cited by the Examiner as teaching the pT7-7 vector which comprises a T7-specific promoter and the *eryAIII* gene of the modular, type I polyketide synthase (PKS) producing erythromycin which gene codes for ketosynthase, acyl transferase, and acyl carrier protein activities, said vector being transformed in *E. coli* for expression.

While not necessarily agreeing the the Examiner's position, in order to expedite prosecution, claims 35, 36, and 38 have been cancelled, making the rejection moot.

### Rejection of Claims Under 35 U.S.C. § 103(a)

Claims 1, 2, and 5 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Lambalot, R.H., *et al.* (J. of Biological Chemistry, 270:24658-24661 (1995)) (hereinafter “Lambalot”), in view of Shen.

Applicant acknowledges that this rejection is limited to the use of ACPS (the *E. coli*-derived holo ACP synthase - a phosphopantetheinylating enzyme) and aromatic, type II PKS genes expressed together in either *E. coli* or Streptomyces, as pointed out by the Examiner.

Lambalot is cited by the Examiner as teaching the *E. coli* gene *dpj*, later called *acpS*, and its gene product, ACPS (a holo-acyl carrier protein synthase), which product “transfers the 4’-phosphopantetheine (4’-PP) moiety from coenzyme A ...to... acyl carrier protein (ACP) in *Eschericia coli*” for activation of an ACP in fatty acid biosynthesis.

The Examiner asserts that Lambalot teaches the overproduction of TcmM (in holo-form in the presence of ACPS) in combination with the heterologous production of the holo ACP synthase, ACPS, in *E. coli*.... The Examiner also states that Lambalot does not teach the heterologous production of a minimal type II PKS....

Shen is cited by the Examiner as teaching the expression of the *tcmJKLMN* genes in a *tcmGHIJKLMNO* null background, and that the *tcmJKLMN* genes encode a minimal type II PKS (KS/AT, ACP, and CLF domains). Applicant respectfully disagrees with the Examiner’s remarks for the reasons described below.

Lambalot describes how holo-ACP synthase (ACPS) transfers the 4’-PP moiety from CoA to Ser-36 of apo-ACP to produce holo-ACP and 3’, 5’-ADP. However, Lambalot states that little has been shown about the mechanism or specificity of this post-translational phosphopantetheinylation process. Lambalot therefore sets forth to study the mechanism and specificity of ACP-phosphopantetheinylation by cloning and overproducing ACPS from *E. coli*. Lambalot reports a 70,000-fold purification and N-terminal sequencing of wild type ACPS that resulted in the identification of the gene that encodes ACPS (*dpj*).

Lambalot “*anticipate[s]* that *dpj* will serve as a valuable tool for the cloning of other ACPSs and will *assist* in the heterologous overproduction of appropriately modified 4’-PP requiring enzymes, such as ... TcmM ..., thereby greatly *facilitating* mechanistic studies of acyl activating enzymes in macrolide, polyketide, depsipeptide, and non-ribosomal peptide biosynthesis, as well as ACP-dependent transacylase activities ” (emphasis added).

Shen does not teach a modified recombinant host cell, which, in unmodified form, does not produce polyketides, which cell is modified to contain an expression system that comprises at least one nucleotide sequence that encodes for a minimal polyketide synthase (PKS). Rather, Shen describes a host cell that in its unmodified form produces the aromatic polyketide tetracenomycin. In addition, since the polyketide synthase proteins described in Shen were isolated from *S. glaucescens*, the PKS would have been already pantetheinylated by the cellular machinery of its host. Thus, there would be no motivation to combine Shen with Lambalot.

According to the MPEP § 2142, three criteria must be met to establish a *prima facie* case of obviousness. (a) there must a suggestion and motivation to modify or combine reference teachings; (b) there must be a reasonable expectation of success, and (c) the cited references must teach or suggest all the claimed limitations.

First, there is no suggestion or motivation either in the references or in the knowledge generally available to one of ordinary skill in the art, to modify or to combine reference teachings. Specifically, there is no suggestion in either reference to place an expression system that comprises at least one nucleotide sequence that encodes for a minimal polyketide synthase (PKS) *and* an expression system that comprises at least one nucleotide sequence that encodes for a holo acyl carrier protein (ACP) synthase into a modified recombinant host cell that in its unmodified form does not produce polyketides. Also, there is no suggestion in Lambalot as to *how* the ACPS will *assist* in the heterologous overproduction of TcmM.

Secondly, a reasonable expectation of success *must* be found in the prior art (*see In re Vaeck*, 947 F.2d. 488, 20 USPQ2d 1438 (Fed. Cir. 1991)). Neither Lambalot or Shen provide any discussion regarding the likelihood of success in placing an expression system that

comprises at least one nucleotide sequence that encodes for a minimal polyketide synthase (PKS) *and* an expression system that comprises at least one nucleotide sequence that encodes for a holo acyl carrier protein (ACP) synthase into a modified recombinant host cell that in its unmodified form does not produce polyketides. In addition, Shen teaches a host cell, which in unmodified form *does* produce a polyketide, specifically tetracenomycin.

In *Interconnect Planning Corp. v. Fiel*, 774 F.2d 1132, 1143, 227 U.S.P.Q. (BNA) 543, 547-48 (Fed. Cir. 1995), the court warned against hindsight and stated “Not only must the claimed invention as a whole be evaluated, but so also must the references as a whole, so that their teaching are applied in the context of their significance to a technician at the time - a technician without our knowledge of the solution.” Since one skilled in the art could not reasonably determine, without hindsight, the successfulness of the claimed invention, it is respectfully submitted that a *prima facie* of obviousness has not been established. It is further submitted that “obvious to try” certain variations is not the standard upon which obviousness is determined properly. *See In re O’Farrell*, 853 F.2d 894, 903, 7 U.S.P.Q.2d 1673, 1680-81 (Fed. Cir. 1988).

In conclusion, Applicants asserts that claims 1, 2, and 5 are patentable under 35 U.S.C. § 103(a) over Lambalot in view of Shen.

Claim 6 stands rejected under 35 U.S.C. § 103(a) as being unpatentable over Lambalot in view of Shen, and in view of Bierman, M., *et al.* (Gene, 116:43-49 (1992)) (hereinafter “Bierman”).

Lambalot and Shen are cited by the Examiner as teaching as described above, and also as not teaching the use of integrating vectors. Bierman is cited by the Examiner as teaching several vectors useful for the integration of a gene of choice into the host cell chromosome via homologous recombination.

Bierman does not remedy any of the deficiencies of Lambalot or Shen described above for claim 1. In addition, Bierman does not provide suggestion or motivation to modify or

combine the references in a manner that would result in the invention as claimed in claim 6, resulting in no reasonable expectation of success. Therefore, since claim 6 depends from claim 1, the arguments provided above for claim 1 also apply to claim 6.

In conclusion, Applicants asserts that claim 6 is patentable under 35 U.S.C. § 103(a) over Lambalot in view of Shen and in view of Bierman.

Claims 8, 9, 11, and 12 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the '146 patent in view of Bierman.

The '146 patent is cited by the Examiner as teaching as described above, and of *not* teaching the option of using modified chromosomes that can result from plasmid integration. Bierman is cited by the Examiner as teaching several vectors useful for the integration of a gene of choice into the host cell chromosome via homologous recombination.

The Examiner asserts that the instant claims are drawn to actinomycetes host cells having (1) at least three vectors, each vector comprising a portion of an aromatic PKS expression system or (2) at least two vectors, each vector comprising a module of a modular PKS expression system; wherein at least one of the vectors is integrated into the host chromosome.

Applicant would like clarify for the Examiner several issues. First, the instant claims are drawn to yeast, *E. coli*, actinomycetes and plant cells (*see* claim 9). Second, Applicant would like to respectfully point out to the Examiner that claim 8 contains the language “a) at least a first and a second vector ...; *or* b) at least one vector and a modified chromosome ...”(emphasis added). Therefore, at least one of the vectors does *not* have to be integrated into the host cell chromosome. Third, claim 11 requires three vectors; vectors one and two each comprising a catalytic region and vector three comprising an ACP activity, and claim 12 requires two vectors, each vector comprising a module of a polyketide synthase.

As described above, claim 8 has been amended to include the phrase “A modified recombinant host cell, which in unmodified form does not produce polyketides, modified to contain either....”

The teachings of the '146 patent have been discussed *supra*. In addition, Bierman does not remedy any of the deficiencies of the '146 patent.

In conclusion, Applicants asserts that claims 8, 9, 11, and 12 are patentable under 35 U.S.C. § 103(a) over the '146 patent in view of Bierman.

Claims 13 and 14 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the '146 patent in view of Oliynk, M., *et al.* (Chemistry and Biology, 3:833-839 (1996)) (hereinafter "Oliynk").

The Examiner states that the instant claims are drawn to host cells having at least two vectors, each vector comprising a module of a modular PKS expression system from a PKS of different origin.

The '146 patent is cited by the Examiner as teaching as described above, and of *not* teaching host cells containing vectors for hybrid modular PKSs. Oliynk is cited by the Examiner as teaching a host cell containing a modular hybrid PKS resulting from the "specific replacement of the entire ... AT1 domain in DEBS1-TE [*eryAI* fused to the TE domain of the *ery* gene cluster] with its ... counterpart from module 2 of the rapamycin-producing PKS".

Since claims 13 and 14 depend from claim 12, which Applicant believes is patentable over the '146 patent for the reasons set forth above, Applicant asserts that claims 13 and 14 are also patentable under 35 U.S.C. § 103(a) over the '146 patent in view of Oliynk.

#### **Rejection Under Obviousness-type Double Patenting**

Claims 1, 3, and 30-39 stand rejected under the judicially created doctrine of obviousness-type double patenting over claims 1, 3, 27, 28, 30-32, and 40-43 of U.S. Patent No. 6,033,883. Claims 35, 36, and 38 have been cancelled.

Claim 2 stands rejected under the judicially created doctrine of obviousness-type double patenting over claim 2 of U.S. Patent No. 6,033,883.

Claim 4 stands rejected under the judicially created doctrine of obviousness-type double patenting over claim 1 of U.S. Patent No. 6,033,883. Claim 4 has been cancelled.

Claims 8, 9, 12-14, and 16 stand rejected under the judicially created doctrine of obviousness-type double patenting over claims 5, 7, 9, 17, and 19 of U.S. Patent No. 6,033,883.

The instant application is a divisional of U.S. Patent No. 6,033,883, and both are commonly owned by Kosan Biosciences. Accordingly, a Terminal Disclaimer is proper to overcome the obviousness-type double patenting rejection, and is enclosed with the Response. Applicant asserts that this Terminal Disclaimer renders the rejections listed above moot.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 300622001610.

Respectfully submitted,

Dated: July 28, 2003

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EXHIBIT A



# A new enzyme superfamily – the phosphopantetheinyl transferases

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**Background:** All polyketide synthases, fatty acid synthases, and non-ribosomal peptide synthetases require posttranslational modification of their constituent acyl carrier protein domain(s) to become catalytically active. The inactive apo-proteins are converted to their active holo-forms by posttranslational transfer of the 4'-phosphopantetheinyl (P-pant) moiety of coenzyme A to the sidechain hydroxyl of a conserved serine residue in each acyl carrier protein domain. The first P-pant transferase to be cloned and characterized was the recently reported *Escherichia coli* enzyme ACPS, responsible for apo to holo conversion of fatty acid synthase. Surprisingly, initial searches of sequence databases did not reveal any proteins with significant peptide sequence similarity with ACPS.

**Results:** Through refinement of sequence alignments that indicated low level similarity with the ACPS peptide sequence, we identified two consensus motifs shared among several potential ACPS homologs. This has led to the identification of a large family of proteins having 12–22 % similarity with ACPS, which are putative P-pant transferases. Three of these proteins, *E. coli* EntD and o195, and *B. subtilis* Sfp, have been overproduced, purified and found to have P-pant transferase activity, confirming that the observed low level of sequence homology correctly predicted catalytic function. Three P-pant transferases are now known to be present in *E. coli* (ACPS, EntD and o195); ACPS and EntD are specific for the activation of fatty acid synthase and enterobactin synthetase, respectively. The apo-protein substrate for o195 has not yet been identified. Sfp is responsible for the activation of the surfactin synthetase.

**Conclusions:** The specificity of ACPS and EntD for distinct P-pant-requiring enzymes suggests that each P-pant-requiring synthase has its own partner enzyme responsible for apo to holo activation of its acyl carrier domains. This is the first direct evidence that in organisms containing multiple P-pant-requiring pathways, each pathway has its own posttranslational modifying activity.

## Introduction

Multienzyme complexes exist for acyl group activation and transfer reactions in the biogenesis of fatty acids, the polyketide family of natural products (e.g. erythromycin and tetracycline), and almost all non-ribosomal peptides (e.g. vancomycin, cyclosporin, bacitracin and penicillin). All of these complexes contain one or more small proteins, ~80–100 amino acids (aa) long, either as separate subunits or as integrated domains, that function as carrier proteins for the growing acyl chain. These acyl carrier protein (ACP) domains, which may be one of the domains of a multi-functional enzyme (in the type I synthases) or a separate subunit (in the type II multienzyme complex synthases), can be recognized by the conserved sequence signature motif (L,V)(G,L)(G,A,F,Y)(D,H,K,E)S(L,Q)(D,A,G) [1]. They are converted from inactive apo-forms

to functional holo-forms by attack of the  $\beta$ -hydroxy sidechain of the conserved serine residue in the ACP signature sequence on the pyrophosphate linkage of coenzyme A (CoASH). This results in transfer of the 4'-phosphopantetheinyl (P-pant) moiety of CoASH onto the attacking serine (Fig. 1). The newly introduced -SH of the P-pant prosthetic group now acts as a nucleophile for acylation by a substrate, which may be acyl-CoA or malonyl-CoA derivatives for the fatty acid and polyketide synthases (PKS), or aminoacyl-AMPs for the peptide and depsipeptide synthetases (Fig. 2). In the PKS complexes the carboxy-activated malonyl-ACP derivative then undergoes decarboxylation, forming a nucleophilic carbanion species that attacks a second acyl thioester to yield a new carbon–carbon bond in one of the steps of polyketide biosynthesis. In peptide and depsipeptide

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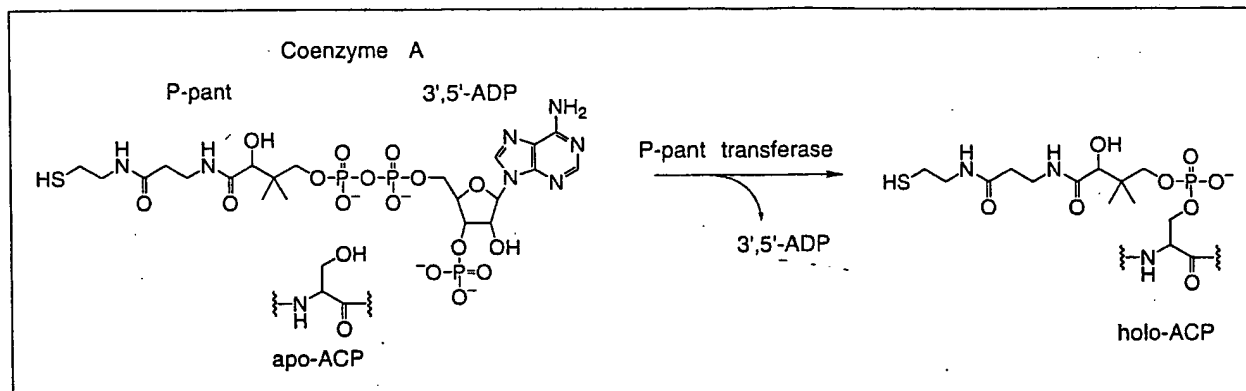
**Key words:** ACP, acyl carrier protein, biosynthesis, non-ribosomal peptide synthetase, phosphopantetheine

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Figure 1



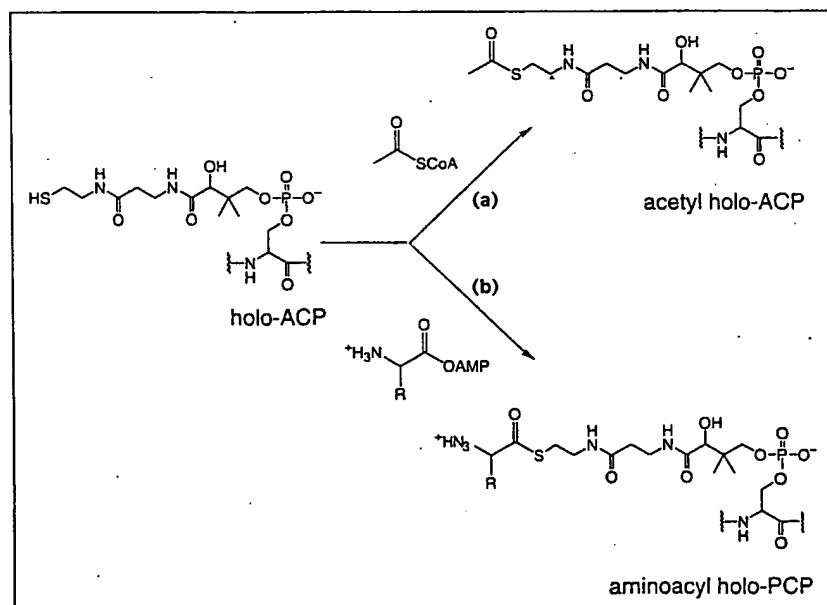
General reaction scheme for posttranslational phosphopantetheinylation. P-pant transferases transfer the 4'-phosphopantetheine moiety

from CoA to a conserved serine residue of apo-ACP to produce holo-ACP and 3',5'-ADP.

synthetases, the aminoacyl-ACPs or hydroxyacyl-ACPs serve as nucleophiles in amide and ester bond-forming steps respectively (Fig. 3). The posttranslational phosphopantetheinylation of apo-ACP domains is clearly essential for the activity of the multienzyme synthetases responsible for the biogenesis of a vast array of natural products. We have therefore searched for and characterized enzymes with P-pant transferase activity. We recently reported the cloning and characterization of the first such transferase, the *Escherichia coli* holo-acyl carrier protein synthase (ACPS), which activates the fatty acid synthase ACP by

converting it to its holo-form [2]. Using the conversion of *E. coli* apo-ACP to holo-ACP as an assay, we purified ACPS 70 000-fold and identified it as the product of a previously described essential *E. coli* gene of unknown function, *dpi* [3]. The *E. coli* ACPS is a 28 kDa dimer of two 125-aa subunits with a  $k_{cat}$  of 80–100 min<sup>-1</sup> and a  $K_M \leq 10^{-6}$  M for apo-ACP. We subsequently showed that the *E. coli* ACPS will also modify apo-forms of several type II ACP homologs including the *Lactobacillus casei* D-alanyl carrier protein (DCP) involved in D-alanylation of lipoteichoic acid [4], the *Rhizobia* protein, NodF, involved

Figure 2



The terminal cysteamine thiol of the phosphopantetheine cofactor acts as a nucleophile for acyl activation. (a) Fatty acid synthases and polyketide synthases transfer acyl groups from acyl-CoA to the phosphopantetheine tether attached to ACP. (b) Non-ribosomal peptide and depsipeptide synthetases first activate their amino-acyl or acyl substrates as their acyl-adenylates before transfer to the phosphopantetheine tether of PCP.

in the acylation of the oligosaccharide-based nodulation factors [5], and the *Streptomyces* ACPs involved in frenolicin, granaticin, oxytetracycline, and tetracenomycin polyketide antibiotic biosynthesis (AMG, RHL and CTW, unpublished results).

The *E. coli* ACPS does not detectably transfer P-pant to the apo-forms of two type I P-pant-requiring proteins involved in amino acid activation, namely apo-EntF which is involved in L-serine activation during *E. coli* enterobactin biosynthesis [6,7] and apo-PCP, a peptidyl carrier protein fragment from the *Bacillus brevis* tyrocidine synthetase (TycA) [8]. Thus other P-pant transferases, specific for the apo-forms of type I peptide synthetases, must exist. Our search in the completely sequenced *Haemophilus influenzae* [9] and *Saccharomyces cerevisiae* genomes for functional homologs of *E. coli* *acpS* initially failed to reveal genes with any apparent homology despite the fact that posttranslational phosphopantetheinylation of ACP domains clearly occurs in these organisms. We report here that more refined database searches yielding peptide sequences with only marginal similarity to ACPS, have in fact led us to identify a large second family of P-pant transferases including the *E. coli* EntD and *B. subtilis* Sfp proteins. The genes encoding these proteins have previously been shown to be required for the production of the non-ribosomal peptides enterobactin and surfactin, respectively (Fig. 4) [10,11]. Putative P-pant transferases have also been identified in *H. influenzae* and *S. cerevisiae* (Fig. 5 and Table 1). We have overproduced and purified EntD, Sfp and a third

*E. coli* protein o195 and have demonstrated the ability of each to catalyze the transfer of 4'-phosphopantetheine from CoASH to apo-protein substrates.

## Results

### Database search for ACP synthase homologs

BLAST searches (basic local alignment search tool) [12] with the 125-aa *E. coli* ACPS protein sequence revealed marginal similarity to the carboxy-terminal region of five fungal fatty acid synthases, suggesting that phosphopantetheinylation activity may have been subsumed as a domain in these polyenzymes (Fig. 5). We propose a scheme, based on several lines of genetic evidence [13–18], in which the carboxyl-terminus of the FAS2 subunit could be responsible for the autophosphopantetheinylation of the amino-terminal ACP domain. However, to date we have been unable to demonstrate P-pant transfer from CoASH to the *S. cerevisiae* FAS2 ACP domain (residues Asp142–Ser230) catalyzed by the putative P-pant transferase domain (residues Gly1774–Lys1894) (data not shown).

Using the small similarity between the fungal FAS2 carboxyl-termini and ACPS as a starting point, we detected potential homology to three bacterial proteins, EntD (*E. coli*), Sfp (*B. subtilis*), and Gsp (*B. brevis*) which have previously been identified as genes that appear to have a common ancestor (orthologous genes) (Fig. 5) [19]. Indeed *E. coli* *entD* and *Bacillus brevis* *gsp* can complement *sfp* mutants, supporting the idea that these three proteins have similar functions [19,20]. The specific biochemical

Figure 3

Acyl-pantetheinyl thioesters have a wide variety of fates in the biosynthesis of complex natural products. Acyl-pantetheinyl thioesters can act as (a) carbanion nucleophiles for carbon skeleton assembly in fatty acid and polyketide biosynthesis or as (b) nitrogen or (c) oxygen nucleophiles to yield amide or ester bonds in peptide and depsipeptide biosynthesis.

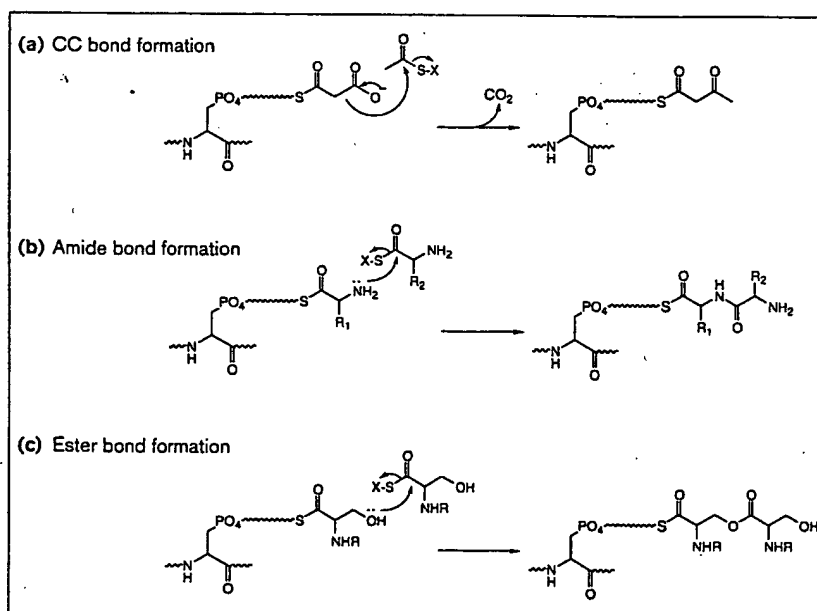
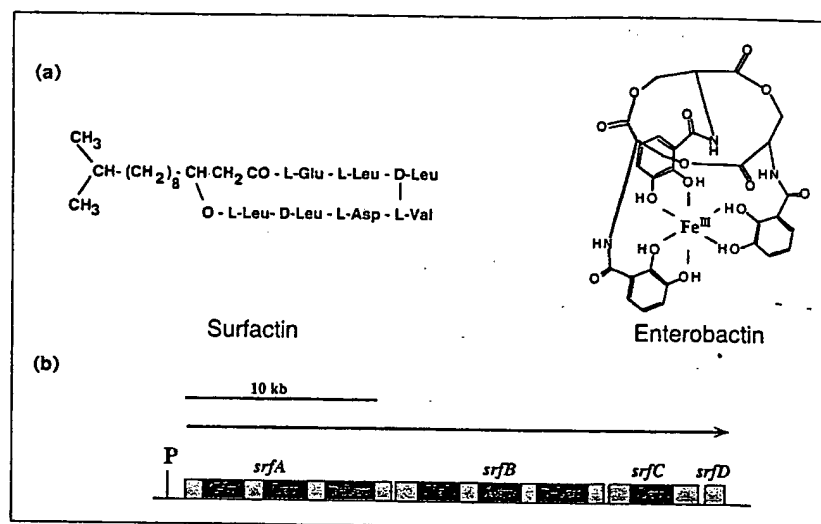


Figure 4



Non-ribosomal peptides and some of the genes involved in their synthesis.

(a) Chemical structures of surfactin and enterobactin. (b) The *srf* operon consists of four open reading frames in which *srfA*, *srfB*, and *srfC* encode for the activities that activate and assemble the seven component amino acids and branched chain  $\beta$ -hydroxy fatty acid of surfactin.

functions of *entD*, *sfp* and *gsp* have up to now remained obscure. *Sfp* was isolated as a locus required for production of the lipopeptide antibiotic surfactin in *B. subtilis* (Fig. 4) [11] and *gsp* is similarly required for gramicidin biosynthesis [19]. Likewise, *entD* has been shown to be required for production of the  $\text{Fe}^{\text{III}}$ -chelating siderophore enterobactin in *E. coli* [10]. Further BLAST searches revealed several other proteins that share potential homology with ACPS (Table 1), including a third *E. coli* open reading frame (in addition to ACPS and EntD) of unknown function designated o195 and proteins involved in cyanobacterial heterocyst differentiation and fungal lysine biosynthesis. Local sequence alignments of the putative P-pant transferase domains reveal two sequence motifs containing several highly conserved residues (Fig. 5, highlighted in yellow).

#### Confirmation of sequence-predicted P-pant transferase activity

To test the sequence-predicted P-pant transferase activity of this enzyme family, we needed to overproduce and purify representative members of this family (EntD, Sfp and o195), prepare apo-forms of putative substrate proteins or subdomains (ACP, PCP, EntF, and SrfB1) and assay the catalytic competence of the putative enzymes.

#### Overproduction, purification and characterization of enzymes

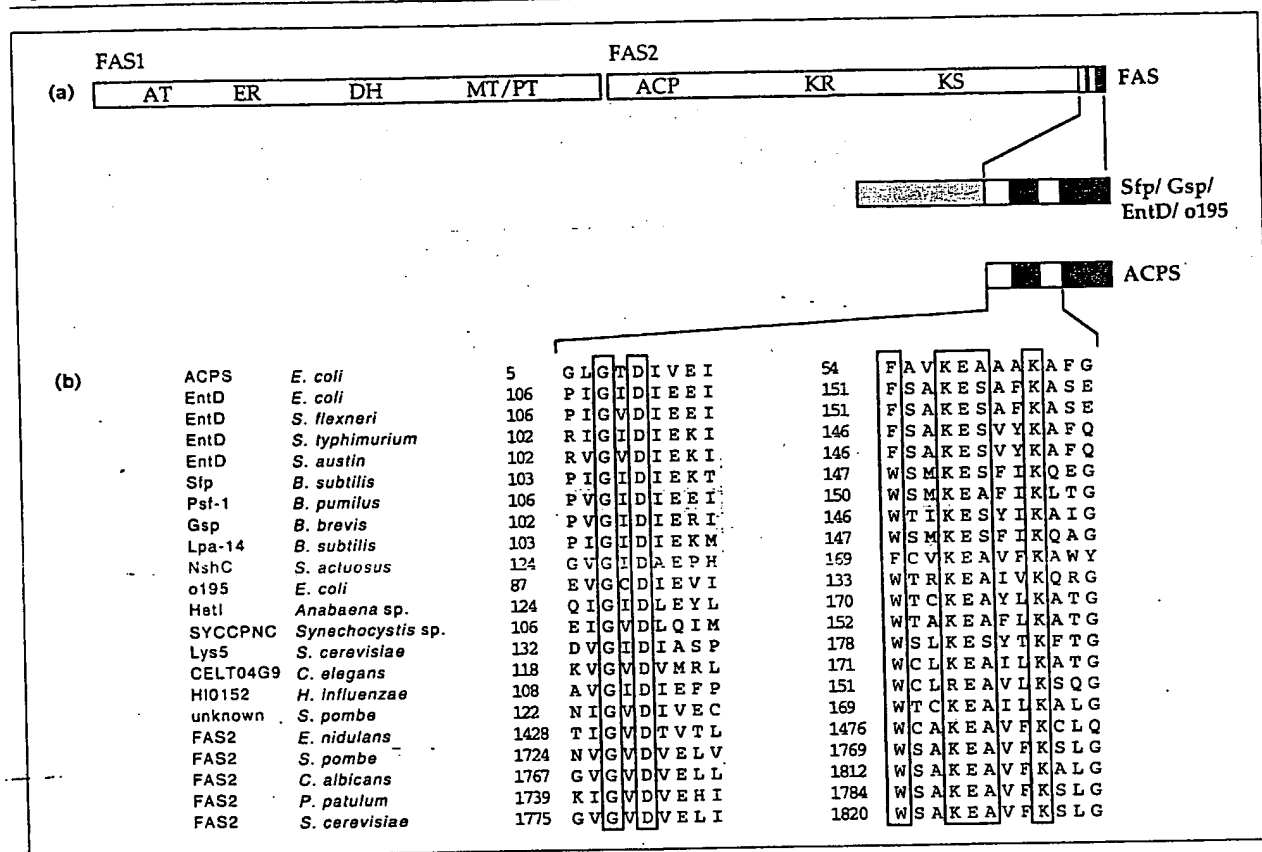
Sfp (26.1 kDa) was overproduced and purified using previously published procedures (Fig. 6) [11]. EntD (23.6 kDa) had previously been cloned, but its overproduction had proven difficult, presumably due to the frequency of rare codons and an unusual UUG start codon [10]. We therefore changed the UUG start to AUG and optimized the codon usage for the first six residues. The *entD* gene

was PCR-amplified from wild type *E. coli* and cloned into the T7-promoter-based pET28b expression plasmid (Novagen). Induction at 25°C yielded soluble EntD, which was purified by ammonium sulfate precipitation and Sephacryl S-100 chromatography. Similarly, the o195 gene was PCR-amplified from wild type *E. coli* cells with codon optimization and cloned into pET28b. Induction at 37°C or 25°C yielded predominantly insoluble o195 protein (21.8 kDa), that could be solubilized in 8 M urea, purified by Q-Sepharose chromatography under denaturing conditions, and renatured by dialysis.

#### Overproduction, purification and characterization of substrates

Apo-ACP and apo-EntF were overproduced and purified as previously described [7] [21]. Apo-PCP (the peptidyl carrier protein of tyrocidine synthetase, see Fig. 7) and apo-SrfB1 (the first amino acid activation and peptidyl carrier protein domains of surfactin synthetase subunit B) were overproduced in *E. coli* and purified as hexahistidine-tagged proteins using nickel chelate chromatography. Typically, when P-pant-requiring enzymes are over-produced in *E. coli* the fraction of recombinant protein that is modified to the holo-form represents only a small percentage of the total recombinant protein [22]. We have been able to confirm that the percentage of holo-ACP present in the purified preparation is below 5% by using analytical HPLC to resolve the apo and holo-forms of the protein (data not shown) [23]. The ratio of apo- to holo-forms of the other substrates after purification was not precisely determined. It is clear, however, as shown below, that sufficient quantities of the apo-forms of each of these proteins were obtained to act as substrates of the P-pant transferase enzymes. P-pant transferase activity toward each of these substrates was assayed by monitoring

Figure 5



The putative phosphopantetheinyl transferase family. (a) Schematic showing location of the proposed P-pant transferase domains (purple) and location of consensus sequences (yellow) in the fungal fatty acid synthases (FAS), the Sfp/Gsp/EntD/o195 homology family, and *E. coli* ACPS. Component FAS activities are abbreviated as AT, acyl

transferase; ER, enoyl reductase; DH, dehydratase; MT/PT malonyl/palmitoyl transferase; ACP, acyl carrier protein; KR, ketoreductase; KS, ketosynthase. (b) Local DNA sequence alignments of the consensus sequences of the P-pant transferase enzyme superfamily. Highly conserved residues are boxed.

the transfer of [ $^3\text{H}$ ]-4'-phosphopantetheine from [ $^3\text{H}$ ]- (pantetheinyl)-CoASH in the presence of the putative P-pant transferase enzyme. Reactions were quenched with 10 % trichloroacetic acid (TCA), and the resulting protein pellet was washed, resolubilized, and counted by liquid scintillation to determine the extent to which the apo-substrate was modified to the holo-form by the covalent attachment of [ $^3\text{H}$ ]-4'-phosphopantetheine.

**Enzymatic activity with apo-ACP and apo-PCP as substrates**  
We were initially concerned that large proteins such as EntF (140 kDa) and SrfB (400 kDa) would be difficult to work with as substrates for the preliminary characterization of the putative P-pant transferases. Indeed our prior attempts to modify purified EntF with ACPS had been unsuccessful (RHL, RSF and CTW, unpublished results). Studies with the large, multifunctional chicken fatty acid synthase had shown that, following partial proteolytic digestion, functional domains representative of

component synthase activities could be isolated [24–28]. Indeed, a functional ACP domain of the rat fatty acid synthase had previously been isolated in this manner (S Smith and VS Rangan, personal communication). By identifying the sequence limits of a peptidyl carrier protein (PCP) domain of tyrocidine synthetase (TycA), Marahiel and coworkers have been able to overproduce a functional 112-aa peptide synthetase carrier protein [8] (Fig. 7). This protein undergoes partial phosphopantetheinylation in *E. coli*, and can then act as an aminoacyl acceptor when incubated with its corresponding adenylation/transferase domain. The PCP substrate is easily purified from endogenous *E. coli* ACP when expressed as a hexahistidine fusion (data not shown). An analogous strategy led to construction and isolation of a hexahistidine fusion of SrfB1, a 143 kDa fragment containing the amino-acid-activating and PCP domains involved in the activation of the fourth residue (valine) in surfactin biosynthesis (Fig. 7).

Table 1

## ACP synthase homologs.\*

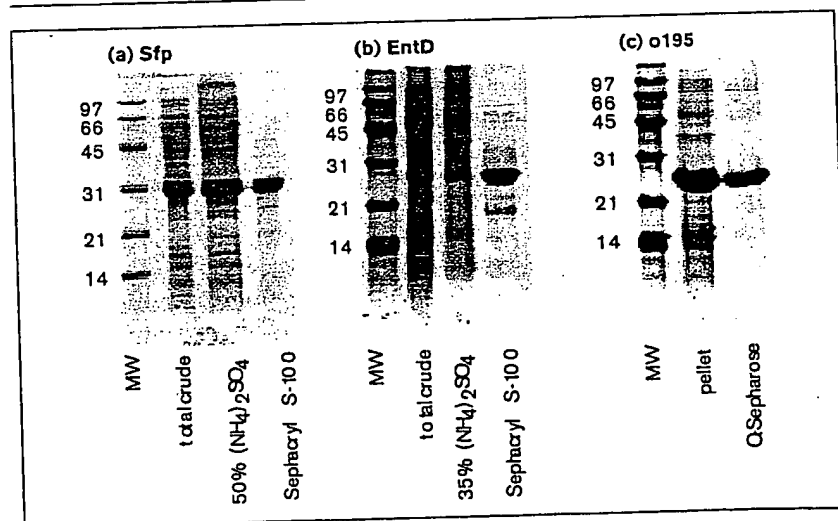
Pathway	Protein	Organism	Size
Enterobactin	EntD	<i>E. coli</i>	209 aa
		<i>S. typhimurium</i>	232 aa
		<i>S. austini</i>	232 aa
		<i>S. flexneri</i>	209 aa
		<i>B. subtilis</i>	224 aa
Surfactin	Sfp	<i>B. pumilus</i>	233 aa
		<i>B. brevis</i>	237 aa
Gramicidin S	Gsp	<i>B. licheniformis</i>	225 aa
Bacitracin	Bli	<i>B. subtilis</i>	224 aa
Iturin A	Lpa-14	<i>S. actuosus</i>	253 aa
Nosiheptide	NshC	<i>S. cerevisiae</i>	272 aa
Lysine	LYS5	<i>E. coli</i>	126 aa
Fatty acids	ACPS	<i>H. influenzae</i>	235 aa
		<i>S. cerevisiae</i>	1894 aa
		<i>C. albicans</i>	1885 aa
		<i>P. patulum</i>	1857 aa
		<i>S. pombe</i>	1842 aa
	HI0152	<i>A. nidulans</i>	1559 aa
	FAS2	<i>Anabaena sp.</i>	237 aa
		<i>Synechocystis sp.</i>	246 aa
Differentiation	HetI	<i>E. coli</i>	195 aa
Unknown	o195	<i>S. pombe</i>	258 aa
		1314154	297 aa
		CELTO4G9	297 aa

\*All sequences except NshC (W Strohl, personal communication, GenBank Accession Number U75434, submitted) and Bli (M Marahiel, unpublished) are available in the GenBank, SwissProt, or EMBL databases.

As mentioned above, recombinant PCP undergoes partial phosphopantetheinylation when expressed in *E. coli* [8]. When recombinant PCP was incubated with purified ACPS and [ $^3$ H]-(pantetheinyl)-CoASH *in vitro*, however, no incorporation of  $^3$ H label was observed (Fig. 8). This result agreed with our earlier finding that ACPS cannot

catalyze the modification of EntF, another type I peptide synthetase component. We therefore hypothesized that another *E. coli* P-pant transferase activity, probably EntD given its sequence similarity to ACPS, is specific for the phosphopantetheinylation of EntF or recombinant PCP overproduced in *E. coli*. To test this idea, we incubated each of the four pure proteins ACPS, EntD, o195, and Sfp with apo-ACP and apo-PCP in the presence of [ $^3$ H]CoASH. Each of the four candidate P-pant transferases generated tritiated ACP and/or PCP in TCA precipitation assays (data not shown). To verify that the  $^3$ H label that coprecipitated with ACP and PCP represented covalent attachment of P-pant, the tritiated products were subjected to SDS electrophoresis and autoradiography (Fig. 8). It is clear that both ACPS and Sfp show robust phosphopantetheinylation activity (Fig. 8a). When apo-ACP is the substrate, EntD is weakly active compared to ACPS and Sfp and o195 is even less active, but both EntD and o195 give signals well above the background, showing that EntD and o195 do have P-pant transferase activity. When the 13 kDa apo-PCP was used as substrate for these four P-pant transferases in Figure 8b, Sfp and EntD are now highly active, but o195 and ACPS give no detectable modification at the single timepoint. When the much larger substrates apo-EntF and apo-SrfB1 fragment (140 kDa) are used (Fig. 8c), the cognate enzymes, EntD for EntF and Sfp for SrfB1, are obviously competent for posttranslational phosphopantetheinylation. Mass spectrometry was used to confirm that the tritium incorporated into the apo-proteins represented transfer of the intact phosphopantetheinyl group. We previously validated this approach using ACPS as catalyst and holo-ACP as product [2] and used it here to examine PCP modification. Mass spectrometric analysis (MALDI-TOF) of unlabeled enzymatic holo-PCP indicated a molecular

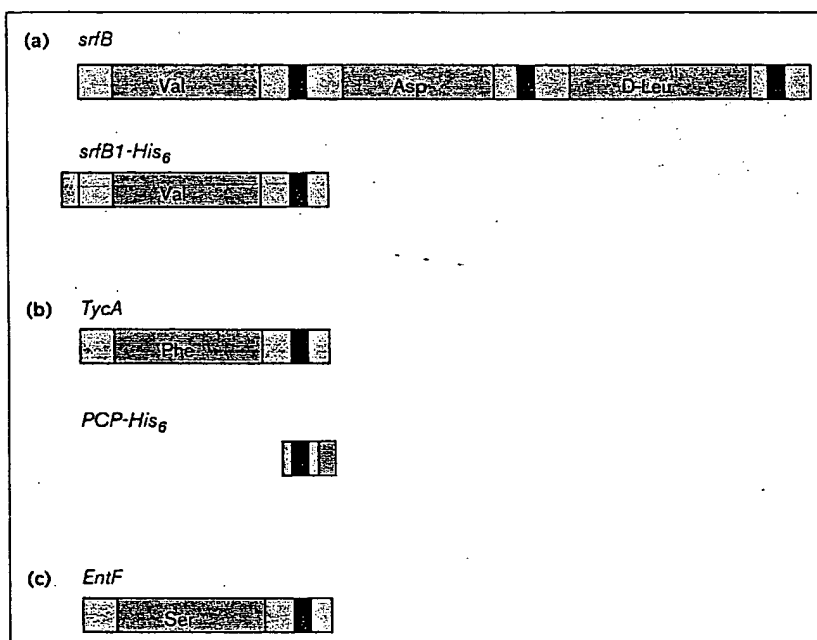
Figure 6



Overproduction of candidate P-pant transferases. (a) Purification of *Bacillus subtilis* Sfp heterologously expressed in *Escherichia coli*. (b) Overproduction and purification of *E. coli* EntD. (c) Overproduction and purification of *E. coli* o195. All gels shown are SDS-PAGE (15 % acrylamide, 2.6 % bisacrylamide).

Figure 7

P-pant acceptor domains and the His<sub>6</sub>-tagged constructs used for purification. Schematic diagram showing the comparative alignment of (a) SrfB and the SrfB1-His<sub>6</sub> fragment, (b) TycA and its constituent PCP domain tagged with His<sub>6</sub> and (c) EntF. Amino-acid-activating domains are shown in light purple. Phosphopantetheine attachment sites are shown in dark purple.



weight of 13 431 (calculated 13 459) in contrast to an observed molecular weight of 13 130 (calculated 13 120) for the apo-PCP substrate. These are the first data that establish that EntD, Sfp, and o195 are enzymes and that they catalyze the transfer of P-pant to the serine sidechain of an acyl carrier protein.

#### Specificity of ACPS, EntD and o195

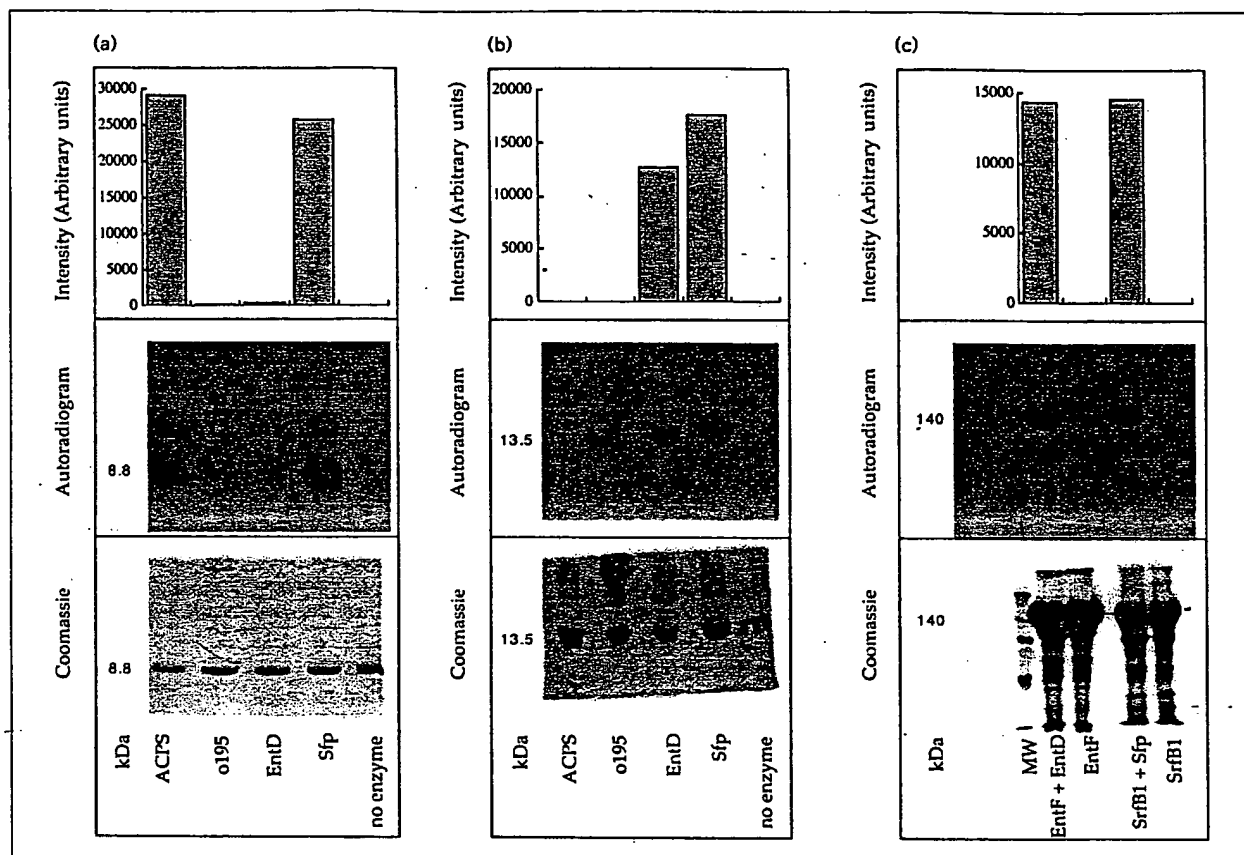
Having demonstrated that EntD does in fact have P-pant transferase activity, we sought kinetic confirmation that it is indeed the enzyme responsible for the posttranslational modification of EntF. As described above, autoradiography of SDS gels confirmed incorporation of radiolabeled phosphopantetheine into EntF catalyzed by EntD (Fig. 8c). Furthermore, a time course of EntD-catalyzed incorporation of radiolabel into EntF provides *in vitro* evidence of at least two partner-specific P-pant transfer reactions occurring within *E. coli*. ACPS specifically catalyzes the transfer of P-pant to apo-ACP, while EntD is the transferase for its partner EntF. EntF is modified effectively by EntD (100 nM), whereas EntF undergoes almost no modification in the presence of 15-fold higher concentrations of ACPS and o195, clearly demonstrating the specificity of EntD for EntF (Fig. 9a). In contrast, apo-ACP is almost exclusively modified by ACPS (Fig. 9b), confirming that in *E. coli* ACPS is the P-pant transferase that activates the type II fatty acid synthase and EntD is the P-pant transferase that activates the type I enterobactin synthetase. The autoradiogram in Figure 8a shows, however, that both o195 and EntD can

modify apo-ACP; the rate of modification is very low, yet is significantly higher than the background rate in the absence of enzyme (Fig. 8a, lane 5). This is presumably due to non-specific enzyme-catalyzed phosphopantetheinylation of the conserved serine residue. Assuming that the inclusion-bound o195 has been properly refolded and that an additional glycine introduced after the methionine start during PCR cloning has no significant effect on activity, it would appear that o195 is specific for a third, as yet unknown, substrate in *E. coli*; presumably P-pant transfer to this unknown protein would require o195 and would not be efficiently catalyzed by ACPS or EntD.

#### Specificity of Sfp toward apo-SrfB1, apo-PCP and apo-ACP

Sfp appears to be non-specific, efficiently catalyzing the modification of the two *Bacillus* derived type I peptide synthetase domains, apo-PCP and apo-SrfB1, the *E. coli* type II fatty acid synthase apo-ACP subunit (Fig. 8) and EntF (data not shown). Based on this evidence, Sfp would appear not to discriminate between type I peptide synthetase domains and type II fatty acid synthase subunits suggesting that there may be crosstalk between Sfp and fatty acid synthase, at least when expressed in *E. coli*. Careful kinetic analysis to determine whether Sfp selectively modifies SrfABC and not the *B. subtilis* fatty acid synthase ACP subunit must await overproduction of the *B. subtilis* ACP, however. Morbidino and co-workers [29] have been able to sequence the entire *B. subtilis* ACP protein by Edman degradation, but the intact *acpP* gene appears to be toxic to *E. coli* and has proven difficult to clone.

Figure 8



P-pant transferase reactions. Coomassie-stained gels are shown for each P-pant transferase incubation with the corresponding autoradiograms and integrated band intensities for individual P-pant transferase incubations. (a) Incubations of ACPS (1.8  $\mu\text{M}$ ), o195 (2.2  $\mu\text{M}$ ), EntD (1.3  $\mu\text{M}$ ), Sfp (1.6  $\mu\text{M}$ ) or no enzyme with apo-ACP

(150  $\mu\text{M}$ ) as substrate. (b) Incubations of ACPS (1.8  $\mu\text{M}$ ), o195 (2.2  $\mu\text{M}$ ), EntD (1.3  $\mu\text{M}$ ), Sfp (1.6  $\mu\text{M}$ ) or no enzyme with apo-PCP (45  $\mu\text{M}$ ) as substrate. (c) Incubations of EntD (1.3  $\mu\text{M}$ ) and Sfp (1.6  $\mu\text{M}$ ) with their homologous substrates apo-EntF and apo-SrfB1.

#### Holo-SrfB1 can activate L-valine

The action of Sfp on the 143 kDa SrfB1 fragment in conversion of the apo-form to the holo-form (Fig. 1) should generate a phosphopantetheinylated SrfB1 competent to undergo specific recognition and acylation by the amino acid L-valine, residue 4 in surfactin (Figs 4,7). Apo-SrfB1 undergoes very little acylation when incubated with [ $^{14}\text{C}$ ]-L-valine, showing that the contamination of this preparation by holo-SrfB1 is small. After incubation with Sfp, however, the level of [ $^{14}\text{C}$ ]-L-valine-holo-SrfB1 covalent complex formed in the complete incubation mixture increases about 14-fold, consistent with an increase in the amount of holo-SrfB1 present. The [ $^{14}\text{C}$ ]-L-valine is used by the amino-acid-activating domain of holo-SrfB1 to make valyl-AMP which then undergoes intramolecular acyl-transfer to the SH group of the P-pant moiety in the adjacent PCP domain. Holo-SrfB1 cannot be covalently acylated by the non-cognate L-aspartate residue, the fifth

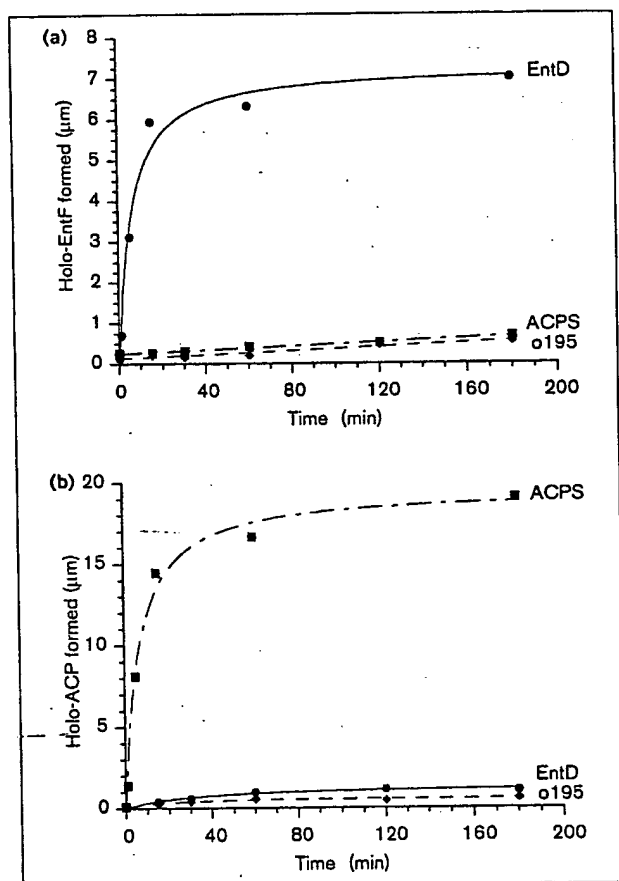
amino acid to be activated by SrfABC, as expected given the absence of an aspartate-specific adenylation domain on SrfB1. Thus the holo-SrfB1 formed following incubation with Sfp and CoASH has both an active adenylation domain and a functional holo-peptidyl carrier protein domain, and should therefore be a useful reagent to probe peptide-bond-forming steps between adjacent sites of multienzyme, multiple thiotemplate syntheses.

#### Discussion

The transfer of 4'-phosphopantetheine from CoASH to conserved serine residues in the signature sequences of acyl carrier protein domains (type I) or subunits (type II) is essential for the functional activation of all fatty acid synthases, polyketide synthases and non-ribosomal peptide synthetase complexes. This posttranslational phosphopantetheinylation introduces a covalently-attached



Figure 9



Time courses of P-pant transferase activity. (a) Time course of EntD (100 nM), ACPS (1.8 μM), or o195 (1.5 μM) incubated with apo-EntF (20 μM) as measured by radioassay. (b) Time course of EntD (1.6 μM), ACPS (100 nM), or o195 (1.5 μM) incubated with apo-ACP (50 μM).

nucleophilic thiol on a long tether that becomes the site of all the initiation and acyl transfer events involved in the assembly of the broad array of natural products synthesized by these enzymes. Thus, identification of the P-pant loading enzymes that create the active holo-ACP forms by posttranslational modification is important to the understanding of both the molecular mechanism of holo-ACP formation and the specificity of serine phosphopantetheinylation. These findings will aid in the design of strategies for heterologous production of functional polyketide and polypeptide synthetases (e.g. in combinatorial biosynthesis of 'unnatural' natural products), and studies aimed at the synthesis of inhibitors of specific P-pant loading reactions (e.g. in fungal lysine biosynthesis, see below).

Our recent purification, characterization, and identification of the *E. coli* holo-ACPS [2] provided the first

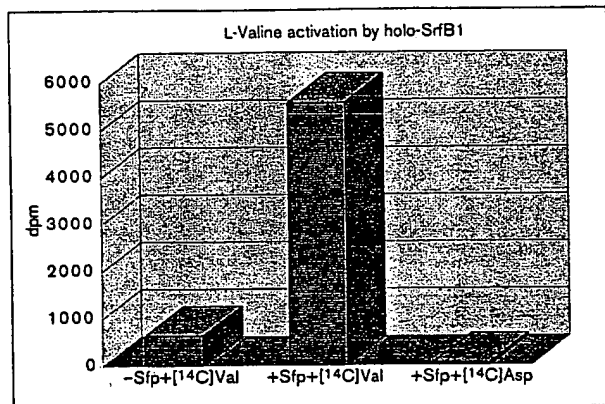
molecular information on this class of posttranslational-modifying enzymes. Somewhat to our surprise, initial database searches with the *E. coli* ACPS sequence revealed no obvious homologs in the protein databases. We eventually detected marginal similarities of 15–22 % over 120 residues in the carboxy-terminal region of three fungal fatty acid synthases (Fig. 5), indicating that the phosphopantetheinylating activity may have been integrated as a domain in these polyenzymes. For example the carboxy-terminal 121 aa of the 1894-aa yeast fatty acid synthase subunit II (yFASII) might act intramolecularly to add a P-pant unit to Ser180 on the putative ACP domain of this polypeptide. We have not yet obtained active fragments of yFASII that catalyze these reactions *in trans*, but Schweizer's group [13–18] has previously reported that two mutated fatty acid synthases, one in which the mutation is at Ser180 and the other at Gly1777, which are inactive alone, can complement each other *in vivo* and *in vitro*, consistent with this proposal.

#### EntD, Sfp and Gsp as specific P-pant transferases

Starting with *E. coli* ACPS, we detected three bacterial proteins EntD, Sfp, and Gsp which have previously been identified by complementation as orthologous genes [19,20]. The specific functions of *sfp*, *gsp* and *entD* have until now been obscure. The studies described here establish that Sfp has phosphopantetheinyl transferase activity and clearly assigns a catalytic loading function to Sfp. It posttranslationally modifies the conserved serine in the first subsite of SrfB, which is responsible for valine activation. We expect that Sfp will be able to modify the consensus serine residue in all seven amino-acid-activating sites in SrfABC (Fig. 4) and by extension that Gsp will catalyze P-pant transfer to the five amino-acid activating sites in GrsA and GrsB, allowing the sequential activation and polymerization of amino acids as required for the thiotemplate mechanism for non-ribosomal peptide bond assembly [30]. The *bli* and *lpa-14* gene products most probably have an equivalent role, that is iterative P-pantetheinylation of each amino acid-activating domain in *B. licheniformis* bacitracin synthetase [31] and *B. subtilis* iturin A synthetase respectively [32]. While *in vitro* enzymatic specificity remains to be fully explored, the *in vivo* genetic studies [11,32] argue strongly for specific partner protein recognition by a distinct P-pant transferase. This may well be a general theme in non-ribosomal peptide antibiotic biosynthesis. While Sfp, Gsp and EntD are required for peptide and depsipeptide biosynthesis, these proteins are not essential for survival [10,33]. We predict, however, that there will be other as yet unidentified P-pant transferases in the *Bacillus* organisms specific for the ACP subunits of their respective fatty acid synthases which, like *E. coli* ACPS, will be essential for viability.

A third example of a partner protein-specific phosphopantetheinyl transferase is EntD, one of the proteins

Figure 10



[<sup>14</sup>C]Valine activation by holo-SrfB1. In the first column, SrfB1 (2  $\mu$ M) was preincubated with CoA (200  $\mu$ M) in the absence of Sfp before subsequent incubation with [<sup>14</sup>C]-L-Valine (100  $\mu$ M, 42.4 Ci mol<sup>-1</sup>) and ATP (2 mM). In the second column, SrfB1 was preincubated with CoA (200  $\mu$ M) in the presence of Sfp (1.3  $\mu$ M) before subsequent incubation with [<sup>14</sup>C]-L-Valine (100  $\mu$ M, 42.4 Ci mol<sup>-1</sup>) and ATP (2 mM). In the third column, SrfB1 (2  $\mu$ M) was preincubated with CoA (200  $\mu$ M) in the presence of Sfp (1.3  $\mu$ M) before subsequent incubation with [<sup>14</sup>C]-L-Aspartate (100  $\mu$ M, 40.3 Ci mol<sup>-1</sup>) and ATP (2 mM).

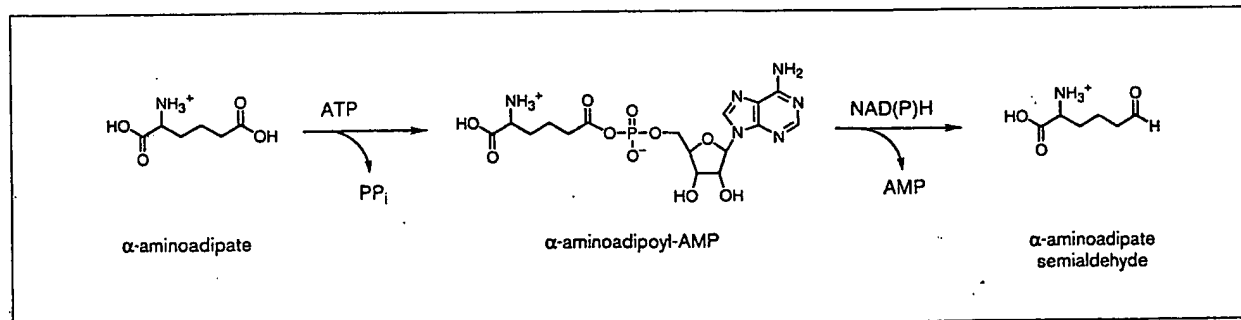
—required for production and secretion of the iron-scavenging dihydroxybenzoyl-serine trilactone enterobactin in *E. coli*. We had previously cloned, sequenced, and purified EntF, a 140 kDa component of the enterobactin synthetase, and demonstrated that it activates L-serine and contains phosphopantetheine [6,7]. As EntD is required for enterobactin biosynthesis *in vivo* [10] and shows high activity for *in vitro* P-pantetheinylation of pure apo-EntF, it is now clear that EntD is defined as the specific P-pant transferase that makes active holo-EntF from apo-EntF *in vivo*. Pure ACPS from *E. coli* will not significantly posttranslationally modify EntF, consistent with the hypothesis that protein-protein recognition

controls the specificity of phosphopantetheinylation *in vivo*. We predict that incubations of EntD and the enterobactin synthetase components with CoASH, L-serine and dihydroxybenzoate should reconstitute *in vitro* enterobactin production. At 140 kDa, EntF is the appropriate size for an amino-acid-activating module in a multidomain polypeptide synthetase [34]. It can be efficiently modified *in vitro* by EntD, showing that P-pant addition can occur after translation of the apo-protein, and not only co-translationally prior to folding of the apo-protein into its native structure. The NMR structure of *E. coli* apo-ACP shows that the nucleophilic Ser36 is in an accessible  $\beta$ -turn [35]; this may be a common architectural scaffolding for ACP domains in polyketide and polypeptide synthases and may be important in recognition by P-pant transferases.

#### Other P-pantetheinyl transferases

Using the EntD/Sfp/Gsp family as a base for further database searches has led to the identification of several additional candidates that are probably P-pant transferase family members (Table 1). Of these, in addition to ACPS and EntD, we have subcloned, expressed and characterized o195 as a third *E. coli* protein with P-pant transferase activity. The activity of o195 towards apo-ACP and apo-EntF is low, suggesting that o195 specifically catalyzes efficient P-pant transfer to an as yet unidentified substrate. A hypothetical protein, HI0152, in *Haemophilis influenzae* has been identified as a putative P-pant transferase. This resolves the apparent problem that no P-pant transferase in the *Haemophilis* genome had previously been found using ACPS-based searches. HI0152 is positioned directly upstream of the *H. influenzae* fatty acid synthase gene cluster, consistent with the notion that its protein product might be involved in fatty acid biogenesis. There is also some evidence that two additional proteins in cyanobacteria have similar functions (Table 1). In *Anabaena*, the genes *HetI*, *HetM*, and *HetN* have been implicated in the production of an unidentified secondary metabolite that inhibits heterocyst differentiation (a

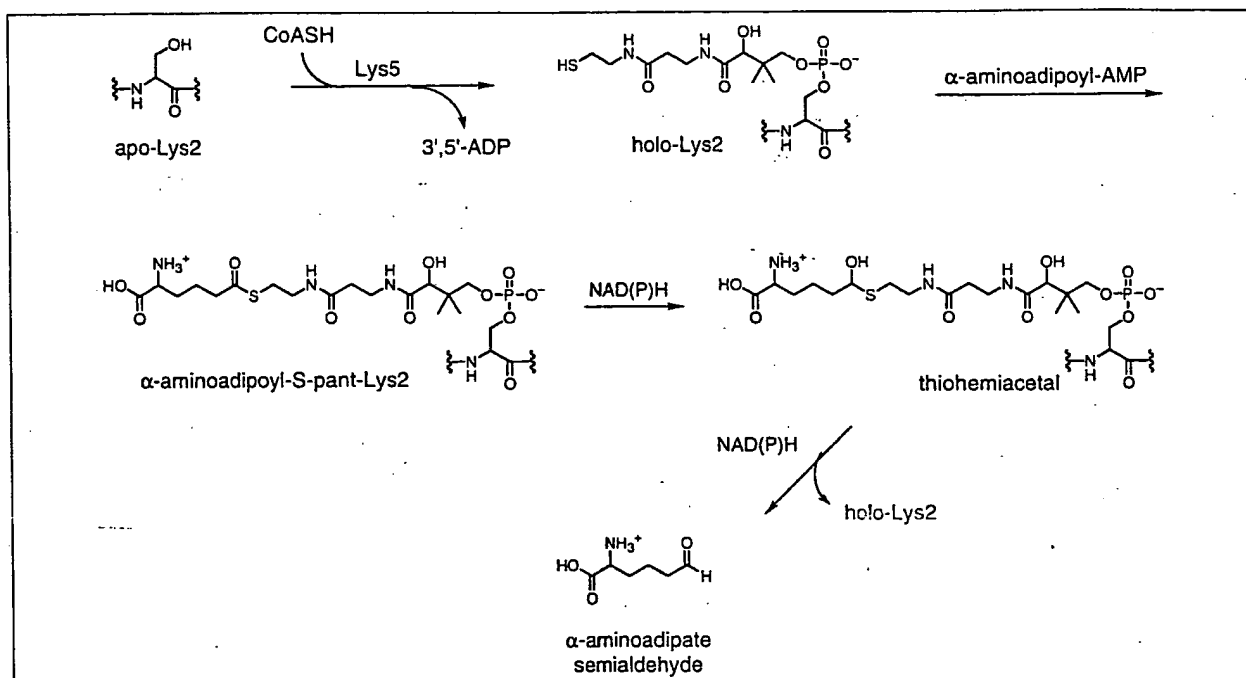
Figure 11



Scheme showing the reaction previously proposed to be catalyzed by the Lys2-Lys5 complex.  $\alpha$ -Aminoadipate is first activated to  $\alpha$ -amino-

adipoyl-AMP. This acyl-adenylate would then undergo direct reduction in a NAD(P)H dependent reaction to yield  $\alpha$ -aminoadipate semialdehyde.

Figure 12



Scheme showing the reaction we now propose to be catalyzed by Lys2. Following phosphopantetheinylation of Lys2 catalyzed by Lys5, aminoadipate is transferred from aminoadipoyl-AMP to yield α-amino-

adipoyl-S-pant-Lys2. This thioester then undergoes direct reduction in a NAD(P)H dependent reaction to yield a thiohemiacetal intermediate which then decomposes to the α-aminoadipate semialdehyde.

process occurring under low fixed nitrogen conditions in which a subset of cyanobacterial cells differentiate into the specialized heterocysts which have the ability to fix nitrogen) [36]. Sequence analysis suggests HetN is a NAD(P)H-dependent oxidoreductase like those involved in the biosynthesis of polyketides and fatty acids, while HetM has an ACP domain. HetI shows similarity to Sfp/Gsp/EntD, and is thus likely to be the HetM-specific phosphopantetheinyl transferase in the synthesis of the hypothesized secondary metabolite.

A final example is the 272-aa Lys5 protein involved in the yeast lysine biosynthetic pathway. Yeast and other fungi synthesize lysine via the unique α-aminoadipate pathway, an eight-step pathway beginning with homocitrate and proceeding via α-aminoadipate to saccharopine to lysine [37]. Complementation analysis suggests that Lys2 and Lys5 are involved in the same step in this pathway, the reduction of α-aminoadipate to aminoadipate semialdehyde [38]. Labeled pyrophosphate exchange experiments indicate that this reaction appears to proceed through an α-aminoadipoyl-AMP intermediate [39,40]. Recent sequence analysis [41] shows Lys2 to be a 155 kDa protein with homology to amino-acid-activating peptide synthetases including TycA, GrsAB, and SrfA. Like these peptide synthetases, Lys2 is believed to cleave

ATP to AMP and PP<sub>i</sub>, activating α-aminoadipate to the α-aminoadipoyl-AMP which is then reduced by NADPH to the aldehyde (Fig. 11). A search for a consensus P-pant attachment site in Lys2 reveals the signature motif LGGHS around Ser880. We therefore propose, in contrast to previous suggestions, that Lys2 and Lys5 may form a two-subunit enzyme [38], that the 272-aa Lys5 is a specific phosphopantetheinyl transferase for Ser880 in Lys2. The thiol of the newly-introduced P-pant prosthetic group on Lys2 would attack the aminoadipoyl-AMP to give aminoadipoyl-S-pant-Lys2, in a similar manner to the sequential formation of aminoacyl-AMP to aminoacyl-S-pant-TycA in the homologous tyrocydine synthetase A subunit (Fig. 12). At this point, hydride addition to the acyl-S-pant-Lys2 would yield a thiohemiacetal which would readily decompose to aldehyde product and HS-pant-Lys2. This sequence has precedent in the reverse direction in the oxidation of glyceraldehyde-3-P to the acyl-S-enzyme in GAP dehydrogenase catalysis via a cysteinyl-S-enzyme hemithioacetal [42].

### Significance

We have obtained evidence for a family of more than a dozen proteins with catalytic posttranslational modification activity. We anticipate that all these proteins will prove to be phosphopantetheinyl transferases with

CoASH as a common substrate but will show specificity, directed by protein-protein interactions, for the conserved serine motif in particular partner proteins. It is likely that most, if not all, of the multienzyme peptide synthetases that use the multiple thiotemplate scaffolding strategy to make peptide antibiotics nonribosomally [30] will have a partner-protein-specific posttranslational modifying enzyme that covalently adds the swinging arm thiol group required to enable acyl transfers. The new proteins in this family are 50–150 amino acid residues longer than the first one discovered, the 125-aa *E. coli* ACPS subunit; these extra amino acids may be responsible for specificity of partner-protein binding. It remains to be seen whether the many polyketide synthase complexes will use this strategy for posttranslational modification.

## Materials and methods

### Overproduction, purification and characterization of EntD, Sfp, and o195

*B. subtilis* Sfp was overproduced and purified from *E. coli* strain MV1190/pUC8-sfp as previously described by Nakano *et al.* [11] (Fig. 6). EntD was PCR-amplified from wild-type *E. coli* K-12 by colony PCR using the forward primer 5'-ATTATATCCATGGgTCCcGTTC-cAAcATGGTCGATAGAAACTACGCA-3' and the reverse primer 5'-TGATGTCAAGCCTTATTAATCGTGTGGCACAGCGTTAT-3' (IDT). The forward primer introduced an *NcoI* restriction site (underlined) which allowed mutation of the TTG start to an ATG start and inserted a Gly codon (GGT) after the Met initiator. In addition the forward primer optimized codon usage for the first six codons of the *entD* gene (modified bases shown in lower case). The reverse primer incorporated a *HindIII* restriction site (underlined). The *NcoI/HindIII* digested PCR product was cloned into pET28b (Novagen) and transformed into competent *E. coli* DH5 $\alpha$ . The recombinant *entD* sequence was confirmed by DNA sequencing (Dana-Farber Molecular Biology Core Facility, Boston, MA). Competent cells of the overproducer strain *E. coli* BL21(DE3) were then transformed with the supercoiled pET28b-*entD*. Induction of a 2-l culture of BL21(DE3)pET28b-*entD* with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) followed by growth at 25°C for 5 h yielded predominantly inclusion-bound EntD, although a modest amount of the overproduced protein was soluble. The overproduction of soluble EntD may be complicated by the fact that the wild type Ent proteins are synthesized in detectable quantities only under iron-starved conditions. Furthermore, although the recombinant EntD is functional as a soluble protein, the wild type EntD has been reported to be membrane bound [43]. The induced cell paste was resuspended in 50 mM Tris, 1 mM EDTA, 5 % glycerol, pH 8.0 (40 ml) and lysed by two passages through the French press at 15 000 psi. Cellular debris and inclusion bound protein was removed by centrifugation at 8000  $\times$  g for 30 min. Pulverized ammonium sulfate was added to 35 %, 65 % and 80 % saturation. The 35 % fraction containing the largest fraction of EntD was applied to a 2.5  $\times$  115 cm Sephacryl S-100 column. The column was eluted at a flow rate of 1 ml min<sup>-1</sup> using the same buffer as above, collecting 8 ml fractions to obtain homogeneous protein.

Similarly, o195 was PCR-amplified from wild-type *E. coli* K-12 by colony PCR using the forward primer 5'-ATTATATCCATGGgTACcGGATAGTTCGGGAAAGTT-3' and the reverse primer 5'-TGATGTCAA GCTTATCAGTTAACTGAATCGATCCATTG-3' (IDT). The forward primer with its *NcoI* restriction site (underlined) gave insertion of a Gly codon (GGT) after the Met initiator codon of the o195 sequence; codon usage for the succeeding codon was also optimized (base change shown in lower case). The reverse primer incorporated a *HindIII* restriction site (underlined). The *NcoI/HindIII*-digested PCR product was cloned into pET28b (Novagen) and transformed into competent

*E. coli* DH5 $\alpha$ . The recombinant o195 sequence was confirmed by DNA sequencing (Dana-Farber Molecular Biology Core Facility, Boston, MA). Competent cells of the overproducer strain *E. coli* BL21(DE3) were then transformed with the supercoiled pET28b-o195. Induction of a 2-l culture (2  $\times$  YT media) of BL21(DE3)pET28b-o195 with 1 mM IPTG followed by growth at 37°C for 3.5 h yielded predominantly inclusion-bound o195 protein. The cell paste was resuspended in 50 mM Tris-HCl, 1 mM EDTA, 5 % glycerol, pH 8.0 (40 ml) and lysed by two passages through a French pressure cell at 15 000 psi. Cellular debris and inclusion-bound protein was pelleted by centrifugation at 27 000  $\times$  g for 30 min. The inclusion-bound protein pellet was resuspended in 30 ml of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 % glycerol and incubated for 30 min at room temperature with 10 mg lysozyme and 30 mg deoxycholate. The pellet was reobtained by centrifugation for 15 min at 27 000  $\times$  g and solubilized in 30 ml of 8 M urea, 50 mM Tris-HCl, pH 8.0, 10 mM dithiothreitol (DTT). Residual solid material was removed by centrifugation for 15 min at 27 000  $\times$  g. The urea-solubilized solution (30 ml) was then applied to a 2.5  $\times$  10 cm Q-Sepharose column equilibrated with 8 M urea, 50 mM Tris-HCl, pH 8.0. The column was washed with 50 ml of the equilibration buffer and then a gradient of 250 ml 0–0.25 M NaCl in 8 M urea, 50 mM Tris-HCl pH 8.0 followed by 200 ml of 0.25–1 M NaCl in the same buffer was applied. The o195 protein eluted at ~200 mM NaCl as determined by 15 % SDS-PAGE. The purified o195 was renatured by diluting a portion of it 10-fold in 8 M urea, 50 mM Tris-HCl, pH 8.0, 10 mM DTT and dialyzing overnight at 4°C against 10 mM Tris-HCl, pH 8.0, 1 mM DTT. Two liters of culture grown in 2  $\times$  YT media yielded 3.1 g of cells from which ~80 mg of o195 protein was obtained.

### Production of apo-protein substrates, apo-ACP, apo-PCP, apo-EntF, and apo-SrfB

The *E. coli* fatty acid synthase ACP was overproduced and purified in its apo-form from *E. coli* strain DK554 [21] following the procedure of Rock and Cronan [44] with the exception that following cell disruption and centrifugation (30 min at 28 000  $\times$  g), the crude extract containing 10 mM MgCl<sub>2</sub> and 10 mM MnCl<sub>2</sub> was incubated for 60 min at room temperature. In this manner, minor amounts of holo-ACP were hydrolyzed to the apo-form using the endogenous *E. coli* ACP phosphodiesterase [45]. The PCP domain of TycA was overproduced with a hexahistidine tag using *E. coli* strain SG13009(pREP4)/pQE60-PCP [8]. Following lysis of the induced culture the His<sub>6</sub>-tagged protein was purified by nickel-chelate chromatography. *E. coli* apo-EntF was purified as previously described [7].

Apo-SrfB1 was cloned from plasmid p120-21E [46]. Briefly, p120-21E was digested with EcoRV to release a 3648-base-pair fragment encoding the SrfB1, valine-activating domain of surfactin synthetase. This fragment was inserted into *StuI*-cleaved pPROEX-1 (Gibco/BRL Life Sciences Technologies) to give plasmid pML118 which codes for a amino-terminal His<sub>6</sub>-tagged SrfB1 domain (142.7 kDa). His<sub>6</sub>-SrfB1 was overproduced using *E. coli* strain AG1574 (courtesy A. Grossman) [47]. Cells were grown at 25°C in 2  $\times$  YT media (2 l) to an O.D. of 0.4 at which point they were induced with 1 mM IPTG and allowed to grow for an additional 4 h. Cells were harvested by centrifugation (3 g), resuspended in 35 ml of 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9 and lysed by two passages through a French pressure cell. This crude extract was clarified by centrifugation for 30 min at 27 000  $\times$  g. More than 50 % of the overproduced SrfB1 was obtained in the soluble fraction as determined by 6 % SDS-PAGE. His<sub>6</sub>-tagged SrfB1 was purified on His-Bind resin (Novagen) following the manufacturer's recommendations.

### Assay for apo-protein to holo-protein conversion by <sup>3</sup>H-P-pant group transfer from <sup>3</sup>H-coenzyme A

P-pant transferase activity (Fig. 1) was measured by radioassay. Enzyme preparations (final enzyme concentrations of 0.1–2.2  $\mu$ M) were incubated with 75 mM Tris-HCl, pH 8.8, 10 mM MgCl<sub>2</sub>, 25 mM DTT, 200  $\mu$ M [<sup>3</sup>H]-[pantetheinyl]-CoASH (5.3  $\times$  10<sup>6</sup> dpm total activity)

and substrate (apo-ACP, apo-PCP, apo-EntF or apo-SrfB1, at final concentrations of 10–150  $\mu\text{M}$ ) for various times at 37° C in a final volume of 100  $\mu\text{l}$ . The incubations were quenched with 10 % TCA and 500  $\mu\text{g}$  bovine serum albumin (BSA) was added as a carrier. The protein was precipitated by centrifugation, washed 3 times with 10 % TCA, and the protein pellet solubilized with 150  $\mu\text{l}$  1 M Tris base. The resuspended protein was added to 3 ml liquid scintillation cocktail and the amount of [ $^3\text{H}$ ]-phosphopantetheine incorporated into the substrate protein was quantified by liquid scintillation counting. Assays for autoradiography were performed as described above except 20  $\mu\text{M}$  [ $^3\text{H}$ ]-[pantetheinyl]-CoASH (2.6  $\times 10^6$  dpm total activity) was used in the assay, no BSA was added to the TCA precipitate, and pellets were solubilized in SDS or native PAGE sample buffer titrated with 1 M Tris base. Assays using apo-PCP as substrate were resolved by 15 % SDS-PAGE, assays using *E. coli* ACP were resolved by 20 % native PAGE, and assays using SrfB1 or EntF were resolved on 8 % SDS-PAGE. Gels were Coomassie-stained, soaked for 30 min in Amplify (Amersham), dried at 80° C under vacuum and exposed to X-ray film for 24–150 h at –70° C (Fig. 8). The autoradiograms were scanned using a digital scanner and relative intensities of the radiolabeled bands were quantified using NIH Image 1.59 software (National Institutes of Health, USA).

#### Assay for activation of L-valine by holo-SrfB1

Apo-SrfB1 (2  $\mu\text{M}$ ) was incubated with 200  $\mu\text{M}$  CoASH, 75 mM Tris-HCl pH 8.0, 10 mM  $\text{MgCl}_2$ , 25 mM DTT and 1.3  $\mu\text{M}$  Sfp for 15 min at 37° C to generate holo-SrfB1. To the SrfB1-Sfp reaction mixture, [ $^{14}\text{C}$ ]-labeled amino acid (valine, 42.4 Ci  $\text{mol}^{-1}$ ; aspartic acid, 40.3 Ci  $\text{mol}^{-1}$ ) was added to 100  $\mu\text{M}$  final concentration. ATP was added to a final concentration of 2 mM and the reaction (115  $\mu\text{l}$ ) was incubated for 15 min at 37° C, then stopped by the addition of 800  $\mu\text{l}$  10 % TCA with 15  $\mu\text{l}$  of a 25 mg  $\text{ml}^{-1}$  BSA solution as carrier. The precipitate was collected by centrifugation, washed with 10 % TCA, dissolved in 150  $\mu\text{l}$  Tris base, and then counted by liquid scintillation.

#### Acknowledgements

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EXHIBIT B

LEXSEE 331 F 3D 860

**INTEGRA LIFESCIENCES I, LTD. and THE BURNHAM INSTITUTE, Plaintiffs–  
Cross Appellants, and TELIOS PHARMACEUTICALS, INC., Plaintiff–Appellee, v.  
MERCK KGaA, Defendant–Appellant, and THE SCRIPPS RESEARCH INSTITUTE  
and DR. DAVID A. CHERESH, Defendants.**

02-1052, 02-1065

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

331 F.3d 860; 2003 U.S. App. LEXIS 11335; 66 U.S.P.Q.2D (BNA) 1865

June 6, 2003, Decided

**SUBSEQUENT HISTORY:** As Corrected July 10, 2003.

**PRIOR HISTORY:** [\*1] Appealed from: United States District Court for the Southern District of California. Senior Judge James M. Fitzgerald, District of Alaska. *Integra LifeSciences I, Ltd. v. Merck KgaA*, 1999 U.S. Dist. LEXIS 10380 (S.D. Cal., Feb. 9, 1999)

**DISPOSITION:** AFFIRMED-IN-PART, REVERSED-IN-PART, and REMANDED.

**CORE TERMS:** patent, peptide, exemption, patented, experiment, cyclic, infringement, patentee, royalty, technology, invention, common law, license, hypothetical, infringing, cell, safe harbor, negotiation, generic, embrace, clinical, sequence, scientific, candidate, testing, specification, linear, experimentation, expiration, infringed

**LexisNexis (TM) HEADNOTES– Core Concepts:**

*Civil Procedure > Appeals > Standards of Review > De Novo Review*  
*Patent Law > Infringement > Claim Interpretation*

[HN1] The United States Court of Appeals for the Federal Circuit reviews a district court's statutory interpretation and patent claim construction without deference.

*Civil Procedure > Appeals > Standards of Review > Substantial Evidence Rule*

[HN2] Determining a reasonable royalty is an issue of fact, which the United States Court of Appeals for the Federal Circuit reverses only in the absence of substantial evidence.

*Civil Procedure > Trials > Judgment as Matter of Law*  
*Civil Procedure > Appeals > Standards of Review*

*> De Novo Review*

[HN3] The United States Court of Appeals for the Federal Circuit reviews the denial of a motion for judgment as a matter of law following a jury verdict without deference.

*Patent Law > Remedies > Bad Faith Enforcement*

[HN4] See 35 U.S.C.S. § 271(e)(1).

*Patent Law > Remedies > Bad Faith Enforcement*

[HN5] 35 U.S.C.S. § 271(e) permits pre-market approval activity conducted for the sole purposes of sales after a patent's expiration.

*Patent Law > Remedies > Bad Faith Enforcement*

[HN6] See 35 U.S.C.S. § 271(a).

*Patent Law > Remedies > Bad Faith Enforcement*

[HN7] The 35 U.S.C.S. § 271(e)(1) safe harbor covers those pre-expiration activities reasonably related to acquiring approval of a drug already on the market by the United States Food and Drug Administration (FDA). A district court thus correctly confines application of the § 271(e)(1) exemption to activity that would contribute to information the FDA considers in approving a drug.

*Patent Law > Remedies > Damages*

[HN8] After finding patent infringement, a jury may award a patentee damages adequate to compensate for the infringement, but in no event less than a reasonable royalty for the use made of the invention by the infringer. 35 U.S.C.S. § 284.

*Patent Law > Remedies > Damages*

[HN9] A reasonable royalty calculation envisions and ascertains the results of a hypothetical negotiation between the patentee and the infringer at a time before the infringing activity began. Thus, the reasonable royalty calculus assesses the relevant market as it would have developed before and absent the infringing activity. Although an exercise in approximation, that analysis



must be based on sound economic and factual predicates. Royalties, like lost profits, are compensatory damages, not punitive.

**Patent Law > Remedies > Damages**

[HN10] The first step in a reasonable royalty calculation is to ascertain the date on which the hypothetical negotiation in advance of infringement would have occurred. The correct determination of that date is essential for properly assessing damages.

**COUNSEL:** Donald R. Dunner, Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P., of Washington, DC, argued for defendant-appellant Merck KGaA. With him on the brief were Thomas H. Jenkins, David A. Manspeizer, and Rachel H. Townsend. Of counsel on the brief were M. Patricia Thayer, Heller Ehrman White & McAuliffe, LLP, of San Francisco, California; and William C. Rooklidge, Howrey Simon Arnold & White, LLP, of Irvine, California. Of counsel was Esther H. Lim, Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P., of Washington, DC.

Mauricio A. Flores, Campbell & Flores LLP, of San Diego, California, argued for plaintiffs-cross appellants Integra LifeSciences I, Ltd. and The Burnham Institute. With him on the brief was David M. Beckwith. Of counsel on the brief were Raphael V. Lupo, Mark G. Davis, and Natalia V. Blinkova, McDermott, Will & Emery, of Washington, DC. Of counsel was Donna M. Tanguay.

**JUDGES:** Before NEWMAN, RADER, and PROST, Circuit Judges. Opinion for the court filed by Circuit Judge [\*2] RADER. Concurring-in-part, dissenting-in-part opinion filed by Circuit Judge NEWMAN.

**OPINIONBY: RADER**

**OPINION:** RADER, Circuit Judge.

Following a jury trial, the United States District Court for the Southern District of California ruled that Merck KGaA (Merck) infringed U.S. Patent Nos. 4,988,621 ('621 patent), 4,792,525 ('525 patent), 5,695,997 ('997 patent), 4,879,237 ('237 patent), and 4,789,734 ('734 patent), belonging to Integra Lifesciences I, Ltd., the Burnham Institute and Telios Pharmaceuticals, Inc. n1 (Integra). The district court held that subsection (e) of 35 U.S.C. § 271 did not immunize Merck against liability for infringement of the '525, '237, '997, and '734 patents. See 35 U.S.C. § 271(e)(1) (2000). The district court, however, granted Merck's motion for summary judgment of invalidity of claim 2 of the '621 patent. *Integra LifeSciences I Ltd. v. Merck KGaA*, 1999 U.S. Dist. LEXIS 10380, 50 USPQ2d

1846, 1850, 1999 WL 398180 (S.D. Cal. 1999). The jury awarded a reasonable royalty of \$15,000,000. Because the district court correctly construed the claims and determined that Merck's infringing activity did [\*3] not fall within the safe harbor of § 271(e), this court affirms those aspects of the district court's order. Because substantial evidence does not support the jury's reasonable royalty award, however, this court remands for further consideration of damages.

n1 As of December 1996, Integra acquired all of Telios' property rights in the asserted patents.

I.

Integra owns the '621, '525, '997, '237, and '734 patents, all of which are related to a short tri-peptide segment of fibronectin having the sequence Arg-Gly-Asp (in single-letter notation, referred to as the "RGD peptide"). The RGD peptide sequence promotes cell adhesion to substrates in culture and in vivo. The RGD sequence promotes this beneficial cell adhesion by interacting with avB3 receptors on cell surface proteins called integrins. In sum, the RGD sequence attaches to the avB3 receptors on the surface of cells. This bond adheres the cells to the substrate containing RGD. In theory, inducing better cell adhesion and growth should promote wound [\*4] healing and biocompatibility of prosthetic devices. In addition, blood vessels grow new branches due to controlled interactions with integrins.

Dr. David Cheresh, a scientist at Scripps, discovered that blocking avB3 receptors inhibits angiogenesis, the process for generating new blood vessels. Inhibiting angiogenesis showed promise as a means to halt tumor growth by starving rapidly dividing tumor cells. Similarly, anti-angiogenic therapies might also treat diabetic retinopathy, rheumatoid arthritis, psoriasis, and inflammatory bowel disease.

Merck recognized the importance of Dr. Cheresh's discovery, and hired Scripps and Dr. Cheresh to identify potential drug candidates that might inhibit angiogenesis. Dr. Cheresh's research showed that cyclic peptide EMD 66203 displayed good inhibition of avB3 receptors. Merck then entered into an agreement with Scripps to fund the "necessary experiments to satisfy the biological bases and regulatory (FDA) requirements for the implementation of clinical trials" with EMD 66203 or a derivative thereof. The agreement contemplated commencing clinical trials with a drug candidate within three years.

Scripps' research led to the discovery of EMD [\*5] 85189, and then EMD 121974—both derivatives of EMD



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66203. Scripps scientists conducted several *in vivo* and *in vitro* experiments "to evaluate the specificity, efficacy, and toxicity of EMD 66203, 85189 and 121974 for various diseases, to explain the mechanism by which these drug candidates work, and to determine which candidates were effective and safe enough to warrant testing in humans." In particular, these tests assessed the action of the cyclic RGD peptides, including the histopathology, toxicology, circulation, diffusion, and half-life of the peptides in the bloodstream. These tests also examined the proper mode of administering the peptides for optimum therapeutic effect. In 1997, the Scripps research team chose EMD 121974 as the best candidate for clinical development.

Integra learned of the Scripps-Merck agreement. Believing the angiogenesis research was a commercial project that infringed its RGD-related patents, Integra offered Merck licenses to the patents-in-suit. After lengthy negotiations, Merck declined. Integra then sued Merck, Scripps, and Dr. Cheresch. Merck answered that its work with Scripps falls under the safe harbor afforded by 35 U.S.C. § 271(e)(1). [\*6] Merck also contended Integra's patents were invalid. Before trial, Integra limited its request for monetary damages to Merck's alleged infringement, and sought only a declaratory judgment against Scripps and Cheresch. After the close of all evidence, the district court granted Scripps' and Dr. Cheresch's motion to dismiss Integra's claim for declaratory judgment.

At trial, the jury found Merck liable for infringing the '525, '997, '237, and '734 patents. The district court determined that the exemption of § 271(e)(1) did not embrace the infringing activity between 1994 and 1998. n2 The district court, however, granted Merck's summary judgment motion on claim 2 of the '621 patent. The district court invalidated this claim based on anticipation by a 1984 Nature article. The parties filed various post-trial motions. In particular, Merck filed motions for JMOL before and after jury deliberations, asserting, *inter alia*, that the accused experiments were exempt from infringement under 35 U.S.C. § 271(e)(1); that Integra did not prove infringement of any patents; and that substantial evidence did not support the damages award. The district court denied [\*7] Merck's motions.

n2 In her dissent, Judge Newman takes this opportunity to restate her dissatisfaction with this court's decision in *Madey v. Duke*. *Madey v. Duke Univ.*, 307 F.3d 1351, 64 USPQ2d 1737 (Fed. Cir. 2002). However, the common law experimental use exception is not before the court in the instant case. The issue before the jury was whether the infringing pre-clinical experiments are immunized

from liability via the "FDA exemption," i.e., 35 U.S.C. § 271(e)(1). The district court did not instruct the jury on the common law research exemption with respect to the Merck's infringing activities. On appeal, Merck does not contend that the common law research exemption should apply to any of the infringing activities evaluated by the jury. Neither party has briefed this issue to this court. Moreover, during oral arguments, counsel for Merck expressly stated that the common law research exemption is not relevant to its appeal. Judge Newman's dissent, however, does not mention that the Patent Act does not include the word "experimental," let alone an experimental use exemption from infringement. See 35 U.S.C. § 271 (2000). Nor does Judge Newman's dissent note that the judge-made doctrine is rooted in the notions of *de minimis* infringement better addressed by limited damages. *Embrex v. Service Eng'g Corp.*, 216 F.3d 1343, 55 USPQ2d 1161 (Fed. Cir. 2000) (Rader, J., concurring); see also *Deuterium Corp. v. United States*, 19 Cl.Ct. 624, 631, 14 USPQ2d 1636, 1642 (Cl. Ct. 1990) ("This court questions whether any infringing use can be *de minimis*. Damages for an extremely small infringing use may be *de minimis*, but infringement is not a question of degree.").

[\*8]

Merck timely appeals, asserting error in the district court's interpretation of § 271(e)(1), in claim construction, and in the refusal to reconsider the amount of the damages award. Integra cross-appeals the denial of its motion for declaratory judgment of infringement by Scripps and Dr. Cheresch, the invalidity finding on the '621 patent, and the court's refusal to enhance the damages award. This court has exclusive jurisdiction. 28 U.S.C. § 1295(a)(1) (2000).

## II.

[HN1] This court reviews statutory interpretation without deference. *Vectra Fitness, Inc. v. TNWK Corp.*, 162 F.3d 1379, 1381, 49 USPQ2d 1144, 1146 (Fed. Cir. 1998). Similarly, this court reviews claim construction without deference. *Cybor Corp. v. FAS Techs., Inc.*, 138 F.3d 1448, 1456, 46 USPQ2d 1169, 1172 (Fed. Cir. 1998) (en banc). [HN2] Determining a reasonable royalty is an issue of fact, which this court reverses only in the absence of substantial evidence. *Unisplay, S.A. v. Am. Elec. Sign Co.*, 69 F.3d 512, 517, 36 USPQ2d 1540, 1544 (Fed. Cir. 1995); *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1240-41, 9 USPQ2d 1913, 1924 (Fed. Cir. 1989). [\*9] Finally, [HN3] this court



reviews the denial of JMOL following a jury verdict without deference. *SIBIA Neurosciences, Inc. v. Cadus Pharm. Corp.*, 225 F.3d 1349, 1354, 55 USPQ2d, 1927, 1930 (Fed. Cir. 2000).

A.

35 U.S.C. § 271(e)(1) defines a safe harbor against patent infringement:

[HN4] It shall not be an act of infringement to make, use, offer to sell, or sell within the United States or import into the United States a patented invention (other than a new animal drug or veterinary biological product (as those terms are used in the Federal Food, Drug, and Cosmetic Act and the Act of March 4, 1913) which is primarily manufactured using recombinant DNA, recombinant RNA, hybridoma technology, or other processes involving site specific genetic manipulation techniques) solely for uses reasonably related to the development and submission of information under a Federal law which regulates the manufacture, use, or sale of drugs or veterinary biological products.

This provision entered title 35 in 1984 as part of the Drug Price Competition and Patent Term Restoration Act of 1984, Pub. L. No. 98-417, 98 Stat. 1585 (1984) (the 1984 Act). The 1984 Act [\*10] had two purposes. In the first place, the 1984 Act sought to restore patent term to pharmaceutical inventions to compensate for the often-lengthy period of pre-market testing pending regulatory approval to sell a new drug. These regulatory delays can deprive a patentee of many years of its patent's term. The second reason for the 1984 Act responded to this court's decision in *Roche Products, Inc. v. Bolar Pharmaceutical Co.*, 733 F.2d 858, 221 USPQ 937 (Fed. Cir. 1984). Specifically, the Act sought to ensure that a patentee's rights did not de facto extend past the expiration of the patent term because a generic competitor also could not enter the market without regulatory approval. See *Eli Lilly & Co. v. Medtronic, Inc.*, 496 U.S. 661, 669-70, 110 L. Ed. 2d 605, 110 S. Ct. 2683 (1990). Thus, the 1984 Act permitted those competitors to conduct experiments in advance of the patent expiration as long as those activities were reasonably related to securing regulatory approval. As previously noted by this court, [HN5] "section 271(e) permits premarket approval activity conducted for the sole purposes of sales after patent expiration." *Hoechst-Roussel Pharms., Inc. v. Lehman*, 109 F.3d 756, 763, 42 USPQ2d 1220, 1226 (Fed. Cir. 1997). [\*11]

The House Committee that initiated this provision expressly described the pre-market approval activity as "a limited amount of testing so that generic manufacturers can establish the bioequivalency of a generic substitute." H.R. Rep. No. 857, at 8, reprinted in 1984 U.S.C.C.A.N. at 2692. The Committee further characterized the limits of this provision, noting that the "nature of the interference with the rights of the patent holder" would not be "substantial," but "de minimus [sic]." *Id.* at 2692, 2714 (stating that "all that the generic can do is test the drug for purposes of submitting data to the FDA for approval. Thus, the nature of the interference is de minimus [sic]"). Thus, the 1984 Act was "designed to benefit the makers of generic drugs, research-based pharmaceutical companies, and not incidentally the public." *Glaxo, Inc. v. Novopharm, Ltd.*, 110 F.3d 1562, 1568, 42 USPQ2d 1257, 1262 (Fed. Cir. 1997).

This court has had occasion to consider the limits of the words "solely for purposes reasonably related to the development and submission of information under a Federal law." Applying this language, this court has permitted clinical trials and [\*12] demonstrations of medical devices under § 271(e)(1). See *Intermedics Inc. v. Ventritex Co.*, 775 F. Supp. 1269 (N.D. Cal. 1991), *aff'd*, 991 F.2d 808, 26 USPQ2d 1524 (Fed. Cir. 1993) (citing *Teletronics Pacing Sys., Inc. v. Ventritex, Inc.*, 982 F.2d 1520 (Fed. Cir. 1992)).

This court has not considered the question arising in this case, namely, whether the pre-clinical research conducted under the Scripps-Merck agreement is exempt from liability for infringement of Integra's patents under § 271(e)(1). The Scripps-Merck experiments did not supply information for submission to the United States Food and Drug Administration (FDA), but instead identified the best drug candidate to subject to future clinical testing under the FDA processes. Thus, this court must determine whether the § 271(e)(1) safe harbor reaches back down the chain of experimentation to embrace development and identification of new drugs that will, in turn, be subject to FDA approval.

[HN6] According to 35 U.S.C. § 271(a), anyone who "without authority makes, uses, sells, or offers to sell any patented invention, within the United States during [\*13] the term of the patent therefor, infringes the patent." 35 U.S.C. § 271(a) (2000). In this case, Merck used the Integra inventions, and thus infringed its patents. Merck may, nonetheless, escape liability for patent infringement if its uses of the Integra inventions fall within the strict limits of § 271(e)(1). To qualify for exemption n3, Merck must show its activities were "solely for uses reasonably related to the development



and submission of information" to the FDA. 35 U.S.C. § 271(e)(1).

n3 While the express language of § 271(e)(1) states that "it shall not be an act of infringement" to carry out research activities "solely for uses reasonably related" to FDA submissions, the statute has been coined an "exemption" in the case law, drawing from terminology used in the legislative history. See H.R. Rep. No. 857, at 5, reprinted in 1984 U.S.C.C.A.N. at 2689 ("In order to facilitate this type of testing, section 202 of the bill creates general [sic] exception to the rules of patent infringement."); see also *Allergan, Inc. v. Alcon Laboratories, Inc.*, 324 F.3d 1322, 1325-26, 66 USPQ2d 1225, 1227-28 (Fed. Cir. 2003) (discussing § 271(e)(1) as an "exemption" to patent infringement). This decision employs the same terminology.

[\*14]

At the outset, this statutory language strictly limits the exemption "solely" to uses with a reasonable relationship to FDA procedures. The term "solely" places a constraint on the inquiry into the limits of the exemption. The exemption cannot extend at all beyond uses with the reasonable relationship specified in § 271(e)(1).

The 1984 Act further specifies the subject of the reasonable relationship test. The exemption covers uses "reasonably related to the development and submission of information" to the FDA. Thus, to qualify at all for the exemption, an otherwise infringing activity must reasonably relate to the development and submission of information for FDA's safety and effectiveness approval processes. The focus of the entire exemption is the provision of information to the FDA. Activities that do not directly produce information for the FDA are already straining the relationship to the central purpose of the safe harbor. The term "reasonably" permits some activities that are not themselves the experiments that produce FDA information to qualify as "solely for uses reasonably related" to clinical tests for the FDA. Again, however, the statutory language limits the reach [\*15] of that relationship test.

In this case, the Scripps work sponsored by Merck was not clinical testing to supply information to the FDA, but only general biomedical research to identify new pharmaceutical compounds. The FDA has no interest in the hunt for drugs that may or may not later undergo clinical testing for FDA approval. For instance, the FDA does not require information about drugs other than the compound featured in an Investigational New

Drug application. Thus, the Scripps work sponsored by Merck was not "solely for uses reasonably related" to clinical testing for FDA.

The reach of the reasonable relationship test as applied in this case receives further confirmation from the context of the 1984 Act. The meaning of the phrase "reasonably related to the development and submission of information" as set forth in § 271(e)(1) is clearer in the context of the role of the 1984 Act in facilitating expedited approval of a generic version of a drug previously approved by the FDA.

As discussed above, the express objective of the 1984 Act was to facilitate the immediate entry of safe, effective generic drugs into the marketplace upon expiration of a pioneer drug patent. The 1984 [\*16] Act thus permits filing of an ANDA (abbreviated new drug application) to expedite FDA approval of a generic version of a drug already on the market. *Bayer AG v. Elan Pharm. Research Corp.*, 212 F.3d 1241, 54 U.S.P.Q.2D (BNA) 1710 (Fed. Cir. 2000). This expedited approval process requires the generic drug company to perform safety and effectiveness tests on its product before expiration of the patent on the pioneer drug if the generic is to be available immediately upon patent expiration. As noted, however, this court had ruled that those pre-expiration tests infringe the patent on the pioneer drug. *Roche*, 733 F.2d at 858. Therefore, the 1984 Act enacted § 271(e)(1) to create a safe harbor for those pre-expiration tests necessary to satisfy FDA requirements. As also noted, the legislative record shows as well that the 1984 Act narrowly tailored the § 271(e)(1) exemption to have only a de minimis impact on the patentee's right to exclude. Therefore, [HN7] the § 271(e)(1) safe harbor covers those pre-expiration activities "reasonably related" to acquiring FDA approval of a drug already on the market. Within this framework and language of the 1984 Act, [\*17] the district court correctly confined the § 271(e)(1) exemption to activity that "would contribute (relatively directly)" to information the FDA considers in approving a drug. *Intermedics*, 775 F. Supp. at 1280.

The exemption viewed in this context does not endorse an interpretation of § 271(e)(1) that would encompass drug development activities far beyond those necessary to acquire information for FDA approval of a patented pioneer drug already on the market. It does not, for instance, expand the phrase "reasonably related" to embrace the development of new drugs because those new products will also need FDA approval. Thus, § 271(e)(1) simply does not globally embrace all experimental activity that at some point, however attenuated, may lead to an FDA approval process. The safe harbor does not reach any exploratory research that may ratio-



nally form a predicate for future FDA clinical tests.

As noted, the text of § 271(e)(1) limits the exemption "solely" to activities "reasonably related to the development and submission of information" to the FDA. Moreover, the context of this safe harbor keys its use to facilitating expedited approval of patented pioneer drugs [\*18] already on the market. Extending § 271(e)(1) to embrace new drug development activities would ignore its language and context with respect to the 1984 Act in an attempt to exonerate infringing uses only potentially related to information for FDA approval. Moreover, such an extension would not confine the scope of § 271(e)(1) to de minimis encroachment on the rights of the patentee. For example, expansion of § 271(e)(1) to include the Scripps-Merck activities would effectively vitiate the exclusive rights of patentees owning biotechnology tool patents. After all, patented tools often facilitate general research to identify candidate drugs, as well as downstream safety-related experiments on those new drugs. Because the downstream clinical testing for FDA approval falls within the safe harbor, these patented tools would only supply some commercial benefit to the inventor when applied to general research. Thus, exaggerating § 271(e)(1) out of context would swallow the whole benefit of the Patent Act for some categories of biotechnological inventions. Needless to say, the 1984 Act was meant to reverse the effects of Roche under limited circumstances, not to deprive entire categories [\*19] of inventions of patent protection.

Because the language and context of the safe harbor do not embrace the Scripps-Merck general biomedical experimentation, this court discerns no error in the district court's interpretation of 35 U.S.C. § 271(e)(1). This court affirms that aspect of the district court's decision.

#### B.

Merck contends that the district court erred in construing the asserted claims of the '525, '997, and '237 patents to embrace both linear and cyclic RGD peptides. Representative claim 8 of the '525 patent reads:

A substantially pure peptide including as the cell-attachment-promoting constituent the amino acid sequence Arg-Gly-Asp-R wherein R is Ser, Cys, Thr or other amino acid, said peptide having cell-attachment-promoting activity, and said peptide not being a naturally occurring peptide.

The '237 patent claims methods of controlling Arg-Gly-Asp mediated attachments of animal cells to substrates. The '997 patent claims recite methods of altering cell attachment activity by bringing cells into contact with

peptide RGD<sub>X</sub>.

In construing claim 8 of the '525 patent, the district court concluded that the claim "imposes no limitations [\*20] on the three-dimensional structure of the peptides at issue." The district court found the claim's terms, in light of the specification, fully support Integra's contention that claim 8 embraces RGD peptides of any linear or cyclic structure.

Because the patents do not expressly refer to cyclic configurations, Merck would limit the term "peptide" to linear peptides. As the district court noted, however, the term "peptide" is understood in the art to represent "two or more amino acids covalently joined by peptide bonds." By this definition, the general term "peptide" encompasses peptides of differing structural forms. The '525 patent specification refers to RGD peptides as having carboxyl and amino termini without expressly discussing cyclic RGD peptide structures. However, the specification as a whole embraces the claimed RGD peptides in both cyclic and linear conformations. The preparation of cyclic peptides was well known to those of skill in the art in 1984. As the district court correctly noted, the asserted patents need not teach that which is already known. The '525 specification references a journal article authored by Merrifield, which discloses the general knowledge of skilled [\*21] artisans in making cyclic peptides at the filing date of the '525 patent. '525 patent, col. 3, ll. 60-64. The record also includes a declaration from Dr. Dedhar that the Merrifield method makes cyclic peptides. Dr. Dedhar stated that a skilled artisan "would have known from reading the patent specification in 1985 that this recognition site exists on all peptides that contain the Arg-Gly-Asp sequence, including cyclic peptides or peptides containing D-form amino acids."

Merck also contends that the patent applicant, while arguing the patentability of claims to cyclic peptides in a later-filed, unrelated application, admitted that the '525 and '997 patents do not teach cyclic peptides. Specifically, the applicant distinguished the claims of unrelated U.S. Patent No. 5,880,092 to Ruoslahti et al. (the '092 patent) over U.S. Patent No. 4,614,517 (the '517 patent), a divisional of the parent of the '525 and '997 patents. In essence, Merck would limit the reach of the '517 patent due to statements the applicant made in another patent application, not in the application leading to the '517.

The Patent and Trademark Office cited the '517 patent as prior art against the Ruoslahti '092 patent. [\*22] During prosecution of the '092 patent, the applicant stated:



The Examiner argues that the generic peptide taught by Ruoslahti et al. anticipates the claimed peptide. . . . The reference does not disclose a subgenus or species from which a subgenus of peptides can be fashioned. As a result, one skilled in the art would not immediately envisage the claimed peptide because of the very large number of peptides encompassed by the generic teaching of this reference. . . . The reference does not suggest cyclizing a peptide to conformationally stabilize it. As a result, one skilled in the art would not immediately envisage the claimed peptide because the reference does not teach a cyclic Arg-Gly-Asp peptide.

According to Merck, this statement limits the term "peptide" in the '517 patent to non-cyclic structures. In the first place, of course, the '092 patent prosecution does not directly limit the '517 patent. See *Abbott Labs. v. Dey, L.P.*, 287 F.3d 1097, 1104-05, 62 USPQ2d 1545, 1550 (Fed. Cir. 2002) (stating that as between two patent applications sharing an inventor and the same assignee, but having no formal relationship, "the relationship, if any, between [\*23] the '839 and '301 patents is insufficient to render the particular arguments made during prosecution of the '301 patent equally applicable to the claims of the '839 patent"). Those comments arise in a context different from the patentability of the '517 patent. Moreover, the above statement loosely describes the patentee's understanding of the '517/'525/'997 specification, but does not definitively limit the scope of the Integra inventions. These comments in the '092 prosecution evince the patent applicant's understanding that the '525 specification generically teaches "a very large number of peptides." The applicant notes that one of skill in the art would not "immediately envisage" conflict with the '092 invention. This characterization does not compromise the scope of the Integra inventions. The '525 patent is a genus patent. Such genus patents do not estop the applicant from later filing an improvement patent, such as the '517, to claim species with particularly useful properties. See *In re Borah*, 53 C.C.P.A. 800, 354 F.2d 1009, 148 USPQ 213 (CCPA 1966). Accordingly, Merck's reliance on this ambiguous and unrelated prosecution history is misplaced, as it does not [\*24] limit the asserted claims to linear peptides.

Thus, the specification of Integra's invention does not limit the term "peptide" to only a linear structure. As the record indicates, the patentee discloses to those skilled in the art both linear and cyclic peptides. The district court correctly construed the term to have its full ordinary meaning in the art.

### C.

Following its determination of infringement, the jury awarded Integra a reasonable royalty of \$15,000,000. Merck contends this award is not supported by substantial evidence. For the reasons articulated below, this court agrees.

[HN8] After finding patent infringement, a jury may award a patentee "damages adequate to compensate for the infringement, but in no event less than a reasonable royalty for the use made of the invention by the infringer." 35 U.S.C. § 284 (2000). Thus, an injured patentee enjoys at least a reasonable royalty even when unable to show lost profits or an established royalty rate. [HN9] A reasonable royalty calculation envisions and ascertains the results of a hypothetical negotiation between the patentee and the infringer at a time before the infringing activity began. *Riles v. Shell Exploration & Prod. Co.*, 298 F.3d 1302, 1311, 63 USPQ2d 1819 (Fed. Cir. 2002) [\*25] (citing *Hanson v. Alpine Valley Ski Area, Inc.*, 718 F.2d 1075, 1078, 219 USPQ 679, 682 (Fed. Cir. 1983)). Thus, the reasonable royalty calculus assesses the relevant market as it would have developed before and absent the infringing activity. Although an exercise in approximation, this analysis must be based on "sound economic and factual predicates." *Riles*, 298 F.3d at 1311 (citing *Crystal Semiconductor Corp. v. TriTech Microelectronics Int'l, Inc.*, 246 F.3d 1336 (Fed. Cir. 2001); *Shockley v. Arcan, Inc.*, 248 F.3d 1349, 58 USPQ2d 1692 (Fed. Cir. 2001)). Royalties, like lost profits, are compensatory damages, not punitive. See *Riles*, 298 F.3d at 1312.

[HN10] The first step in a reasonable royalty calculation is to ascertain the date on which the hypothetical negotiation in advance of infringement would have occurred. The correct determination of this date is essential for properly assessing damages. The value of a hypothetical license negotiated in 1994 could be drastically different from one undertaken in 1995 due to the more nascent state of the RGD peptide research in 1994. Indeed, factoring in the rapid [\*26] development of biotechnological arts, a year can make a great difference in economic risks and rewards. In any event, the record is not clear on the hypothetical negotiation date.

Integra charged Merck with infringing the '525 patent by conducting various experiments between 1994 and 1998. The district court ruled that some of the 1994 experiments are not infringing acts. This finding, however, does not properly establish the critical hypothetical negotiation date. The record shows that at least one of the 1994 Merck experiments was not considered exempted from infringement due to experimental use. Integra alleged infringement via an August 1994 pharmacokinetic



experiment. Merck represents to this court in its brief that the jury returned a verdict finding that "Scripps and Cheresch infringed all of the patents by conducting various experiments between 1994-98 (the 'accused experiments' or the '1994-98 experiments')." Yet, it is not evident whether the 1994 pharmacokinetic experiment was considered by the jury for infringement purposes. Thus, the record does not clearly indicate whether 1994 or 1995 is the proper date for the first infringement. If indeed the record shows that the [\*27] first infringement occurred in 1994, then the hypothetical negotiation should be regarded as having occurred at least before that earlier date. On remand, the trial court will have the opportunity to clarify the proper timing of the reasonable royalty calculus.

Integra argues further that Mr. Anderson's testimony supports the \$15,000,000 award. Mr. Anderson proffered a hypothetical license figure based, in part, on Merck's 1995 expectations of obtaining FDA approval of a cyclic peptide therapeutic. As already noted, however, if the hypothetical negotiation occurred in 1994, Merck did not have that expectation. Thus, an earlier date will change the risks and expectations of the parties.

Integra argues that the \$15,000,000 award is not excessive because Merck and ImClone executed a license for a pre-clinical stage antibody in 1990. The ImClone license included an up-front fee of \$3,500,000, plus an additional \$14,000,000 over the following three years. The record is not clear, however, that the level of risk associated with the licensed ImClone technology was equally applicable to the RGD technology. While the ImClone agreement may show that Merck pays up-front fees for research [\*28] technology before clinical testing, the nature of the ImClone technology at the time of the Merck agreement may have no bearing on the value of a hypothetical RGD license. At the point before Merck ever attempted its first test on RGD technology, it would have assumed all the risks of failure - either scientific failure to identify a suitable therapeutic candidate or economic failure to market a successful product. If those risks as perceived before any experimentation differed from the risks quantified in the ImClone agreement, then the ImClone example does little to set the value of the pre-clinical RGD research project at a comparable figure. The parties' inability to project success at the pre-clinical research stage of the RGD project weighs heavily in determining a reasonable royalty, particularly if the time for the valuation of the project moves back to 1994. The record does not show that the ImClone licenses occurred under scientific or economic circumstances that permit comparison to this hypothetical RGD license.

Although comparisons to other licenses are inherently suspect because economic and scientific risks vary greatly, the record does seem to contain a more appropriate [\*29] analogue than the ImClone license. In 1995, Telios and Genentech reached agreement to jointly develop and market a product with RGD peptides. This license is somewhat contemporary and involves similar technology. Under the Genentech agreement, Telios agreed to provide Genentech with exclusive patent licenses and years of research services. Under the hypothetical negotiation in this case, Integra would perform no pre-clinical research for Merck. Thus, even this analogue would need revision and inquiry.

The \$15,000,000 royalty also does not appear to take into account numerous factors that would considerably reduce the value of a hypothetical license. For example, Integra purchased Telios (together with all of its products, patents and know-how) for \$20,000,000 in 1996. A \$15,000,000 award figure to compensate for infringement of only some of Telios' patents before Integra's acquisition seems unbalanced in view of the overall acquisition price.

Finally, on remand, the trial court will have the opportunity to consider other factors when sketching an overall picture of a hypothetical negotiation for a license to RGD technology. The value to a licensee of research tools lies, in part, [\*30] in the point at which those tools are employed in the drug development continuum. A research tool enabling the identification of a drug candidate during high throughput screening, for instance, may supply more value to the ultimate invention than a research tool used to confirm an already recognized drug candidate's safety or efficacy. This type of challenge in assessing royalties confronted the district court in *SIBIA*, although this court never reached the issue of damages on appeal. See *SIBIA*, 225 F.3d at 1349, 1352-53; see also Donald Ware, *Research Tool Patents: Judicial Remedies*, 30 *AIPLA Q.J.*, 267, 282-87 (2002). Similarly, the amount Merck would agree to pay for Integra's RGD technology could be influenced by the point of placement of this technology in its drug development process.

In addition, the number of patent licenses needed to develop a drug may also affect the value placed on any single technology used in the development process. The cumulative effect of such stacking royalties can be substantial, particularly when reach-through royalties come into play. See Ware, *supra*, at 295-96. While this court does not opine on [\*31] the applicability of a reach-through royalty in this case, the presence or absence of stacking royalties for research tools may color the character of a hypothetical negotiation between Merck



and Integra for access to the RGD peptide technology. n4 Thus, both the time point at which Merck utilized RGD peptides in its drug development process and the effect, if any, of stacking royalties may also play a role in crafting the hypothetical license between Integra and Merck.

n4 According to the National Institutes of Health (NIH), research tools are defined to be "tools that scientists use in the laboratory, including cell lines, monoclonal antibodies, reagents, animal models, growth factors, combinatorial chemistry and DNA libraries, clones and cloning tools (such as PCR), methods, laboratory equipment and machines." *Sharing Biomedical Research Resources: Principles and Guidelines for Recipients of NIH Research Grants and Contracts*, 64 Fed. Reg. 72,090, 72092 n.1 (Dec. 23, 1999). The dissent asserts that Integra's patented RGD peptides are not research tools, "but simply new compositions having certain uses." Dissent at Section D. The dissent does not explain why one of those "certain uses" cannot embrace use of an RGD peptide as a laboratory tool to facilitate the identification of a new therapeutic. Regardless of whether one considers the RGD peptides to assume the label of a "research tool," the points discussed in relation to determining the value of the peptides during a hypothetical negotiation are valid.

[\*32]

This court gives great deference to the district court's role in assessing the credibility of witnesses and weighing the facts of a given case. Nonetheless, the record evidence does not adequately support the jury's damage award of \$15,000,000 in this case. Therefore, the district court's denial of JMOL as to the damage award is reversed. This case is remanded for further factual development and the calculation of damages consistent with the principles discussed herein.

D.

All other arguments made by the parties have been carefully considered, but are not found persuasive by this court. Thus, this court affirms the district court's denial of

Integra's request for declaratory judgment, its holding that the '621 patent is invalid, and its refusal to grant enhanced damages.

#### CONCLUSIONS

Because the district court properly determined Merck's infringing activities were not "solely for uses reasonably related" to provision of information to the FDA under 35 U.S.C. § 271(e)(1), this court affirms that aspect of the district court's decision. In addition, the district court correctly construed the term "peptide" to have its full ordinary meaning in the art. [\*33] However, the district court erred in denying Merck's motion for reconsideration of an appropriate reasonable royalty. Therefore, the court remands for further consideration of the damages issue.

#### COSTS

Each party shall bear its own costs.

AFFIRMED-IN-PART, REVERSED-IN-PART,  
and REMANDED

CONCURBY: NEWMAN (In Part)

DISSENTBY: NEWMAN (In Part)

DISSENT: NEWMAN, Circuit Judge, concurring in part, dissenting in part.

This case raises a question of the nature and application of the common law research exemption, an exemption from infringement that arose in judge-made law almost two centuries ago, and that recently has come into sharper focus. Its correct treatment can affect research institutions, research-dependent industry, and scientific progress.

The question is whether, and to what extent, the patentee's permission is required in order to study that which is patented. For the Scripps/Merck research, the panel majority holds that all of the activity at Scripps during 1995-1998 was "discovery-based research" and that there is no right to conduct such research, under either the common law research exemption or the statutory immunity established in 35 U.S.C. § 271(e)(1). [\*34] However, neither law nor policy requires that conclusion, and both law and policy have long required a different conclusion in implementation of the purpose of the patent system.

The purpose of a patent system is not only to provide a financial incentive to create new knowledge and bring it to public benefit through new products; it also serves to add to the body of published scientific/technologic knowledge. The requirement of disclosure of the details of patented inventions facilitates further knowledge and understanding of what was done by the patentee, and may lead to further technologic advance. The right to conduct research to achieve such knowledge need not, and should not, await expiration of the patent. That is



not the law, and it would be a practice impossible to administer. Yet today the court disapproves and essentially eliminates the common law research exemption. This change of law is ill-suited to today's research-founded, technology-based economy. I must, respectfully, dissent.

#### A. The Scripps/Merck Activities

The research on which Scripps and Merck collaborated was directed to studies of certain peptide components of fibronectin, containing a chain of the [\*35] three amino acids arginine (R), glycine (G), and aspartic acid (D). The scientists who founded Integra's predecessor, co-plaintiff Telios, discovered that peptides containing this RGD sequence had potential use in promoting wound healing and prosthesis adhesion, and obtained patents on various RGD peptide compositions and methods; however, the Telios scientists were unable to develop a viable commercial product, and eventually sold the patents to Integra.

Merck KgaA of Germany began funding research at Scripps in 1988, after Dr. Cheresh of Scripps had identified a monoclonal antibody, designated LM609, having activity as an inhibitor of integrin n5 activity. The collaboration was enlarged in 1995 with increased funding by Merck, after Dr. Cheresh discovered that a Merck-provided peptide designated EMD66203, having the sequence c(RGDfV), n6 inhibits new blood vessel growth by interaction with a specific integrin. In this collaboration, cyclic RGD peptides of various structures and composition n7 were synthesized and studied, as knowledge was gained concerning their chemical and biological properties and the effects of changes in their structure. It was discovered that the cyclic peptide [\*36] structure solved certain problems that had been experienced with the Telios linear RGD peptides, and that some products have anti-angiogenic properties, of interest for treatment of such diseases as cancer, macular degeneration, rheumatoid arthritis, and others. "Angiogenic" refers to the process of generating new blood vessels, a process essential to tumor growth. As summarized by Merck, Dr. Cheresh testified that the purpose of the research was to "(1) assess the potential efficacy of the peptides as therapeutic agents; (2) discover the mechanism of action of the peptides; and (3) shed light on histopathology, toxicology, circulation, diffusion, and half-life of the peptides in the bloodstream." Brief at 15. The ultimate goal of the research was undisputed: it was to find a product that would be sufficiently effective in the treatment of angiogenic disease that it could be developed and brought to market for this purpose.

n5 "Integrin" refers to a family of cell surface

receptors.

n6 In this nomenclature L isomers of amino acids are represented by their single letter codes in capital letters (R=arginine, G=glycine, D=aspartic acid). The "f" represents the D isomer of phenylalanine. "NMeV," appearing *infra*, represents the N-methyl derivative of valine. The "c" means that the sequence is cyclic.

[\*37]

n7 Also at issue in this litigation was whether the Integra patents cover the cyclic RGD peptides that were produced and investigated by Scripps/Merck. On this close question of infringement I would affirm the district court, as does the panel majority, for there was extensive evidence at trial, including the (conflicting) advice of experts, supporting the district court's findings.

The record describes modifications in the structure of RGD-containing peptides and investigations of their properties in the Scripps/Merck collaboration, including: receptor binding assays to investigate the efficacy and specificity of structural change; angiogenesis/chick CAM assays for inhibition of blood vessel formation in chick embryos when vessel growth is artificially induced, to study the mechanism of action, pharmacokinetics, and other properties; angio-matrigel experiments to investigate inhibition of artificially induced vascularization in mice; cell adhesion assays by spectrophotometric measurement of inhibition of cell attachment to protein, to provide information about mechanisms, efficacy, and other [\*38] properties; chemotaxis studies to determine the effect of various peptides on cell migration over extracellular matrix fibers; use of chick embryos to obtain pharmacokinetic data; fluorescent-activated cell sorting to study the effect on the receptor-ligand binding reaction, to aid in understanding mechanisms of activity; vascularization of the retina and induced arthritis of the joints, studied with mice and rabbits; chick CAM assays to study angiogenesis associated with tumor transplantation and growth in chick embryos; and tumor growth in SCID-mice or nude mice, including studies of mechanism, pharmacology, and pharmacokinetics.

As this research progressed, so did the scientific understanding of these peptide products and their mode of action. In 1997 Scripps/Merck selected the peptide designated EMD 121974 and having the amino acid sequence c(RGDf-NMeV) as the most promising product thus far, although they continued to synthesize and evaluate further modifications of the peptides. In 1998 an



Investigatory New Drug application for EMD 121974 was filed with the Food and Drug Administration.

The panel majority describes all of this activity as "discovery-based research," and holds [\*39] that it is subject to neither a common law research exemption nor the "safe harbor" of § 271(e)(1). I cannot agree. In my view, either the common law research exemption or the development associated with § 271(e)(1) immunity embraces all of these activities.

#### B. The Common Law Research Exemption

The common law research exemption is a limited exception to the patentee's unrestricted right to exclude. Its jurisprudential origin is with Justice Story, who stated in *Whittemore v. Cutter*, 1 Gall. 429, 29 Fed. Cas. 1120, 1121, F. Cas. No. 17600 (C.C.D. Mass. 1813) (No. 17,600), that

it could never have been the intention of the legislature to punish a man who constructed such a machine merely for philosophical experiments,[ n8 ]or for the purpose of ascertaining the sufficiency of the machine to produce its described effects.

Again in *Sawin v. Guild*, 1 Gall. 485, 21 Fed. Cas. 554, F. Cas. No. 12391 (C.C.D. Mass. 1813) (No. 12,391) Justice Story distinguished

the making with an intent to use for profit, and not for the mere purpose of philosophical experiment, or to ascertain the verity and exactness of the specification.

The few judicial decisions on this [\*40] issue have applied the research exemption when no commercial purpose was demonstrated for the research. See, e.g., *Chesterfield v. United States*, 141 Ct. Cl. 838, 159 F. Supp. 371 (Ct. Cl. 1958) (experimentation by the United States did not infringe the patent); *Ruth v. Stearns-Roger Manufacturing Co.*, 13 F. Supp. 697 (D. Colo. 1935) (patent not infringed when the Colorado School of Mines cut up and studied the patented machines).

n8 By "philosophical" experiments Justice Story was referring to "natural philosophy," the term then used for what we today call "science." For example, in the volume on Classification of Subjects of Inventions Adopted by the United States Patent Office, January 1, 1868 (GPO 1868), the section headed "Philosophical Instruments — Class XXV" lists "Philosophical Apparatus, Scales, Measures, and Instruments of Precision."

The majority's prohibition of all research into patented subject matter is as impractical as it is incorrect. The information [\*41] contained in patents is a major source of scientific as well as technologic knowledge. Indeed, in many areas of technology, technical information is not published outside of patent documents. A rule that this information cannot be investigated without permission of the patentee is belied by the routine appearance of improvements on patented subject matter, as well as the rapid evolution of improvements on concepts that are patented.

The subject matter of patents may be studied in order to understand it, or to improve upon it, or to find a new use for it, or to modify or "design around" it. Were such research subject to prohibition by the patentee the advancement of technology would stop, for the first patentee in the field could bar not only patent-protected competition, but all research that might lead to such competition, as well as barring improvement or challenge or avoidance of patented technology. Today's accelerated technological advance is based in large part on knowledge of the details of patented inventions and how they are made and used. Prohibition of research into such knowledge cannot be squared with the framework of the patent law.

The patent statute requires full [\*42] disclosure of the invention, including details of enabling experiments and technical drawings and best modes and preferred embodiments, even commercial sources of special components. Such details would be idle and purposeless if this information cannot be used for 17-20 years. Indeed, there would be little value in the requirement of the patent law that patented information must be removed from secrecy in consideration of the patent right to exclude, if the information is then placed on ice and protected from further study and research investigation. To the contrary, the patent system both contemplates and facilitates research into patented subject matter, whether the purpose is scientific understanding or evaluation or comparison or improvement. Such activities are integral to the advance of technology.

In the framework of United States patent law there is no obligation that the patentee use the invention; the obligation is to disclose it and describe it and to provide enabling detail whereby it can be duplicated without undue experimentation. The patentee's permission is not required whenever a patented device or molecule is made or modified or investigated. Study of patented information [\*43] is essential to the creation of new knowledge, thereby achieving further scientific and technologic progress.



Of course, the common law exemption is not unlimited. Indeed, it is a narrow exemption, for it must preserve the patentee's incentive to innovate, an incentive secured only by the right to exclude. It is the patentee who opened the door by providing the initial knowledge, without which there would be nothing to improve. Setting the boundaries of a common law exemption requires careful understanding of the mechanisms of the creation, development, and use of technical knowledge, and of today's complexity of interactions among invention and the innovating fruits of invention. It is the initial inventor whose rights must receive primary consideration in an effective patent law, for the public interest starts with the threshold invention. However, while that threshold invention may (as here) exact tribute from or enjoin commercial and pre-commercial activity, the patent does not bar all research that precedes such activity.

I do not here undertake to define the boundaries of the research exemption for all purposes and all activities, other than to observe that there is a generally [\*44] recognized distinction between "research" and "development," as a matter of scale, creativity, resource allocation, and often the level of scientific/engineering skill needed for the project; this distinction may serve as a useful divider, applicable in most situations. Like "fair use" in copyright law, n9 the great variety of possible facts may occasionally raise dispute as to particular cases. However, also like fair use, in most cases it will be clear whether the exemption applies. Indeed, the question of boundary does not arise for the Scripps/Merck research here at issue, for the statutory immunity of § 271(e)(1) takes effect wherever the research exemption ends, as I discuss in Part C, post.

n9 The research exemption has been compared to "fair use," which was also a creation of Justice Story, in *Folsom v. Marsh*, 9 Fed. Cas. 342, F. Cas. No. 4901 (C.C.D. Mass. 1841) (No. 4901). The House Report drew this analogy in discussing 35 U.S.C. 271(e)(1), stating: "Just as we have recognized the doctrine of fair use in copyright, it is appropriate to create a similar mechanism in the patent law. That is all this bill does." H.R. Rep. No. 98-857 at 30 (1984), reprinted in 1984 USCCAN 2714.

[\*45]

The Scripps/Merck activities that are here challenged took place during the collaboration outlined in Part A ante. Were all research using RGD peptides prohibited until the Integra/Telios patents expired, not even the patent owner would benefit, for the patented products

had failed in Telios' hands, leaving the patents valueless until Scripps and Merck made their discoveries as to the cyclic peptides and their anti-angiogenic properties. The panel majority states that because the Scripps/Merck research had the goal of curing cancer and commercializing the cure, this purpose moved the research outside of any common law exemption. However, an ultimate goal or hope of profit from successful research should not eliminate the exemption. The better rule is to recognize the exemption for research conducted in order to understand or improve upon or modify the patented subject matter, whatever the ultimate goal. That is how the patent system has always worked: the patent is infringed by and bars activity associated with development and commercialization of infringing subject matter, but the research itself is not prohibited, nor is comparison of the patented subject matter with improved technology [\*46] or with designs whose purpose is to avoid the patent.

### C. Immunity Under § 271(e)(1); Damages

§ 271(e)(1). It shall not be an act of infringement to make, use, [sell or import]. . . solely for uses reasonably related to the development and submission of information under a Federal law which regulates the manufacture, use, or sale of drugs or veterinary biological products.

The panel majority holds that the 35 U.S.C. § 271(e)(1) "safe harbor" does not apply to federal registration of pioneering new drugs like the Scripps/Merck products here at issue, but only to registration of generic copies of drugs for which the patent is about to expire. I agree as to the origin of § 271(e)(1). However, the statute has been interpreted as of broader scope, see *Eli Lilly & Co. v. Medtronic Co.*, 496 U.S. 661, 110 L. Ed. 2d 605, 110 S. Ct. 2683 (1990), and the parties accept that § 271(e)(1) applies to Merck's filing of the Investigatory New Drug application for EMD 121974. This issue was not raised at trial.

The majority also holds that none of the research by Scripps/Merck qualifies for § 271(e)(1) immunity because the research was directed [\*47] to "discovery," not to federal registration. I agree that "the § 271(e)(1) safe harbor [does not] reach back down the chain of experimentation to embrace development and identification of new drugs." Maj. op. at 8. However, the territory that the Scripps/Merck research traversed, from laboratory experimentation to development of data for submission to the FDA, was either exempt exploratory research, or was immunized by § 271(e)(1). It would be strange to create an intervening kind of limbo, between exploratory research subject to exemption, and the FDA



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statutory immunity, where the patent is infringed and the activity can be prohibited. That would defeat the purposes of both exemptions; the law does not favor such an illogical outcome.

After a product loses the § 271(e)(1) protection, it is subject to the full force of any adversely held patents. That aspect is not here in dispute, but it is relevant to the damages verdict, not appropriately treated in the majority opinion.

If the question of damages is remanded, as the panel majority holds, my colleagues go too far in counseling the parties as to how to present their case. The "hypothetical negotiation" is no more than a convenience [\*48] in estimating value, not a compulsory economic standard, and surely not one that requires appellate speculation as to when the parties might have hypothetically negotiated, requiring retrial. The presentation of evidence on damages was extensive, and included evidence that well supported a jury verdict that included a license. Our appellate role is to decide whether the jury verdict was supported by substantial evidence as presented at trial, not to rewrite the trial script as we might have tuned it to our taste. See *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1570, 24 USPQ2d 1401, 1411 (Fed. Cir. 1992) (when there is sufficient evidence to support the jury verdict in light of the entire record, the verdict must stand unless the evidence permits only one reasonable conclusion.) The damages verdict is readily sustainable if construed to include a license for the remaining two to three years of patent life. The evidence before the jury well supports the magnitude of the damages award. I would affirm the verdict on that basis.

#### D. The Research Exemption and Research Tools

The panel majority states that acceptance of a common law research exemption [\*49] would eliminate patents on "research tools." That is a misperception. There is a fundamental distinction between research into the science and technology disclosed in patents, and the use in research of patented products or methods, the so-called "research tools."

A research tool is a product or method whose purpose is use in the conduct of research, whether the tool is an analytical balance, an assay kit, a laser device (as in *Madey v. Duke University*), n10 or a biochemical method such as the PCR (polymerase chain reaction). It is as subject to the patent right as is any other device or

method, whether it is used to conduct research or for any other purpose. Use of an existing tool in one's research is quite different from study of the tool itself.

n10 *Madey v. Duke University*, 307 F.3d 1351 (Fed. Cir. 2002) concerned the use of a patented laser device for the purpose for which it was made, not research into understanding or improving the design or operation of the machine. The facts of *Madey v. Duke* do not invoke the common law research exemption, despite the broad statement in that opinion. I do not disagree with that decision on its facts; I disagree only with its sweeping dictum, and its failure to distinguish between investigation into patented things, as has always been permitted, and investigation using patented things, as has never been permitted.

[\*50]

My colleagues on this panel appear to view the Integra patents as for a "research tool." That is a misdefinition. The RGD-containing peptides of the Integra patents are not a "tool" used in research, but simply new compositions having certain biological properties. The Scripps/Merck syntheses and evaluations of new RGD peptides were not use of the Integra products as a research tool.

The majority states that this issue is not before us, that "the district court did not instruct the jury" on the question. However, the question was before the district court, who held that the common law exemption applied to one Scripps experiment in 1994, but to nothing else. The issue was before the district court, and counsel explained at oral argument that they were not pressing this argument "in part because of a very recent case." Since the question was fundamental to resolution of this case, it cannot be ignored.

#### Conclusion

I do not attempt to resolve, for all technologies and circumstances, the application of the research exemption or the point at which research into patented technology loses the immunity that the common law has always provided. However, the basic research here performed was [\*51] within the common law research exemption, and the development shielded by § 271(e)(1) took up where the research exemption left off. Thus the accused activities were either exempt from or immune from infringement.

EXHIBIT C

lenges ETF's rights as to those goods but seeks to preclude a more expanded line of clothing or services being offered under the VITTORIO RICCI mark.

[3] Nina Ricci has shown that NINA RICCI has become an increasingly strong identification of source, that it has been vigilant in protecting its marks from encroachment by others, that NINA RICCI or related marks have been used on the goods and services at issue since well before ETF's limited use of its mark for such goods and services, that the goods of the parties would be sold in some of the same stores, and that overlap of the goods, which did not exist to any significant extent in prior years and does not, in fact, yet exist to any significant extent, would be fostered if ETF were allowed to expand into the field now occupied by Nina Ricci. Further, we see no basis for holding that Nina Ricci has estopped itself from challenging the right of expansion ETF asserts by reason of its registering NINA RICCI for shoes, a mark ETF did not oppose. We therefore conclude that the opposer, Nina Ricci, has met its burden of demonstrating that there exists a likelihood of confusion between its NINA RICCI and related marks and ETF's VITTORIO RICCI mark for the goods and services in question. In reaching this decision, we must reiterate the teaching of our predecessor court that there is "no excuse for even approaching the well-known trademark of a competitor . . . and that all doubt as to whether confusion, mistake, or deception is likely is to be resolved against the newcomer, especially where the established mark is one which is famous. . . ." *Planter's Nut & Chocolate Co. v. Crown Nut Co., Inc.*, 305 F.2d 916, 924-25, 134 USPQ 504, 511 (CCPA 1962). See also *Specialty Brands, Inc. v. Coffee Bean Distribs., Inc.*, 748 F.2d at 674, 223 USPQ at 1285. Based on the record before the Board, we must view ETF as the "newcomer" to the clothing and fashion accessories business despite the fact that ETF uses the established VITTORIO RICCI mark in the rather limited area of shoes and belts.

Accordingly, the decision of the Trademark Trial and Appeal Board is reversed.

REVERSED

U.S. Patent and Trademark Office  
Board of Patent Appeals and Interferences

Ex parte Mark

No. 88-2811

Decided June 24, 1989

## PATENTS

### 1. Patentability/Validity — Adequacy of disclosure (§115.12)

Claims, for cysteine-depleted muteins of biologically active proteins, which require mutin which is produced to retain biological activity of native protein, are enabling, in view of record establishing that, for given protein having cysteine residues, one skilled in art would be able to routinely determine whether deletion or replacement of cysteine residues would result in mutin which is within claims, and fact that given protein may not be amenable for use in present invention, in that cysteine residues are needed for biological activity of protein, does not militate against conclusion of enablement, since one skilled in art is clearly enabled to perform such work as needed to determine whether cysteine residues of any given protein are needed for retention of biological activity.

### 3. Practice and procedure in U.S. Patent and Trademark Office — Prosecution — Filing date (§110.0906)

Earliest filing date of parent applications to which present claims are entitled under 35 USC 120 is filing date of first parent application in chain which sets forth generic description of synthetic muteins of present invention.

Appeal from rejection of claims (Robin Teskin, examiner; Thomas Wiseman, supervisory patent examiner).

Applicants David F. Mark, Leo S. Lin, Shi-Da Yu Lu, and Alice M. Wang appeal from rejection of claims, application, serial no. 06/698,939 filed Feb. 7, 1985 (cysteine-depleted muteins of biologically active proteins). Reversed.

Albert P. Halluin and Jane R. McLaughlin, Emeryville, Calif., for appellants.

Before Goldstein, Pellman, and W. Smith, examiners-in-chief.

W. Smith, examiner-in-chief.

This is an appeal from the final rejection of claims 1 through 5 and 45 through 69. The appeal as to claims 54 and 56 was withdrawn

by appellants' counsel at oral argument.<sup>1</sup> Thus, claims 1 through 5, 45 through 53, 55 and 57 through 69 remain for our consideration, which are all of the claims remaining in the application.

Claims 1, 45, 54, 55, 56, 57 and 64 are illustrative of the subject matter on appeal and read as follows:

1. A synthetic mutin of a biologically active native protein in which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutin having at least one of said cysteine residues substituted by another amino acid and said mutin exhibiting the biological activity of said native protein.

45. A structural gene having a DNA sequence that encodes a synthetic mutin of a biologically active native protein which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutin having at least one of said cysteine residues substituted by another amino acid and said mutin exhibiting the biological activity of said native protein.

54. A method of preventing a protein having at least one cysteine residue that is free to form a disulfide link from forming said link comprising mutationally altering the protein by deleting the cysteine residue or replacing the cysteine residue with another amino acid.

55. The method of claim 54 wherein the protein is biologically active and the cysteine is not essential to said biological activity.

56. The method of claim 54 wherein the cysteine residue is replaced with serine or threonine.

57. A method for making a gene having a DNA sequence that encodes a synthetic mutin of a biologically active native protein which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutin having at least one of said cysteine residues substituted by another amino acid and said mutin exhibiting the biological activity of said native protein comprising:

<sup>1</sup> It became apparent at oral argument that appellants' invention revolves around the present synthetic muteins retaining the biological activity of the native protein. The method of claims 54 and 56 is not so limited. When this was brought to counsel's attention during oral argument, counsel orally withdrew claims 54 and 56 from appeal.

(a) hybridizing single-stranded DNA comprising a strand of a structural gene that encodes said protein with a mutant oligonucleotide primer that is complementary to a region of said strand that includes the codon for said cysteine residue or the antisense triplet paired with said codon, as the case may be, except for a mismatch with said codon or said antisense triplet which mismatch defines a triplet that codes for said other amino acid;

(b) extending the primer with DNA polymerase to form a mutational heteroduplex; and

(c) replicating said mutational heteroduplex.

64. An oligonucleotide for use in making a structural gene, said gene having a DNA sequence that encodes a synthetic mutin of a biologically active native protein which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, and said mutin having at least one of said cysteine residues substituted by another amino acid and said mutin exhibiting the biological activity of said native protein, by oligonucleotide-directed mutagenesis, said oligonucleotide having a nucleotide sequence that is complementary to a region of the strand of the structural gene that includes the codon for the cysteine residue or the anti-sense triple paired with said codon, as the case may be, except for a mismatch of said codon that defines a triplet that codes for said other amino acid.

The reference relied upon by the examiner is:

Mark et al.

(Mark '584) 4,518,584 May 21, 1985

A reference relied upon by the Board is:

Mark et al.

(Mark '585) 4,588,585 May 13, 1986

The sole rejection of the claims remaining on appeal is under 35 USC §112, first paragraph, as being nonenabled. In support of the rejection, the examiner relies upon a statement of prior art which appears at page 3, lines 22-line 34 of the present specification and at column 1, lines 55-56 of U.S. Patent No. 4,518,584 to Mark et al., one of the present parent applications, which reads as follows:

In this regard Shepard, H.M., et al, *Nature* (1981) 294:563-565 describe a mutant of IFN- $\beta$  in which the cysteine at position 141 of its amino acid sequence

(there are three cysteines in native human IFN- $\beta$  at positions 17, 31, and 141, *Gene* (1980) 10:11-15 and *Nature* (1980) 285:542-547) is replaced by tyrosine. This mutant was made by bacterial expression of a hybrid gene constructed from a partial IFN- $\beta$  cDNA clone having a G  $\square$  A transition at nucleotide 485 of the IFN- $\beta$  gene. The mutin lacked the biological activity of native IFN- $\beta$  leading the authors to conclude that the replaced cysteine was essential to activity.

In addition, the examiner relies upon a statement which appears in an amendment filed in co-pending, commonly assigned Serial No. 06/876,819 which reads as follows:

The review of the newly allowed claims with the inventors in light of the presently available data concerning the claimed species revealed that the seven Cys to Ser substitutions possible within the mature CSF-1 sequence shown in Figure 5 each result in a substantial reduction in the *in vitro* colony stimulation assay specified in claim 53. Thus, the ser<sup>70</sup>CSF-1 species claimed in claim 20 (and also in claims 22 and 29) does not meet the requirement specified by claim 53. Nevertheless, applicants are of the view that the DNA encoding the ser<sup>70</sup>CSF-1 species as well as the other Cys substitution species may have other uses, as experimental probes for example. Accordingly, claim 20 which specifically claims ser<sup>70</sup>CSF-1 has been made independent. For the above described reasons, the ser<sup>70</sup>CSF-1 species has been deleted from claims 22 and 29.

The examiner's rejection is summarized at page 3 of the Examiner's Answer as follows: Essentially, the position taken in the rejection is that it would require undue further experimentation to construct by recombination methods (site specific mutagenesis) the innumerable mutants encompassed by the instant claims (claims encompass modification of any protein which comprises a "non-essential" cysteine residue) and to screen the mutants produced for any of those which exhibit biological activity after modification.

The examiner further reasons that it is generally known in the art that cysteine residues facilitate the proper disulfide bonds and consequently the proper folding of a protein. The examiner concludes that it is likely that most of the mutants prepared by applicants' claimed methodology "would be inoperative simply because the removal of the cysteine would disturb proper folding of the molecule, thereby potentially blocking the active site or

sites of the resulting mutin." (Examiner's Answer, page 4)

The examiner points out on page 6 of her Answer that the claims on appeal encompass any protein, even those which have not been characterized or cloned and that the mere sequencing of all possible proteins encompassed by the claims on appeal, would entail an undue amount of experimentation.

As set forth on page 7 of the Appeal Brief: Appellants' position is that given the disclosure of the present invention substituting a nonessential cysteine with a neutral amino acid, the nonessential cysteine residues of any candidate protein could be identified and substituted in ten days employing the methods disclosed in the instant disclosure and the general knowledge of the art at the time the application was filed. Such limited amount of experimentation based on the disclosure in the application and the success shown by three proteins certainly does not constitute undue experimentation.

These arguments are supported by the declaration of co-appellant Alice M. Wang filed under 37 CFR 1.132 on August 10, 1987. In her declaration Ms. Wang sets forth what she terms a reasonable scheme for determining which cysteine residues in a generic biologically active protein would be available for substitution without destroying the biological activity. The declaration sets forth a step-by-step scheme for implementing the claimed invention which parallels the disclosure of the present application.

The examiner sets forth on page 5 of her Answer that Ms. Wang's declaration does not refute the determination that undue experimentation is needed for implementation of the claimed invention because of the "limited successful embodiments shown and the established unpredictability associated with such modifications as to how many such site-specific mutageneses would need to be undergone to obtain even one alternative biologically active mutin."

[1] We have carefully considered the respective positions of the examiner and the appellants and find that we agree with appellants that the claims remaining on appeal are enabled by the present disclosure. The working examples of the present specification set forth experiments which establish that three proteins, IFN- $\beta$ , IL-2 and TNF, have non-essential cysteine residues which may be deleted or replaced with the resulting mutin retaining the biological activity of the native protein. When it is considered that the claims remaining on appeal all require that the mutin produced retain the biological

activity of the native protein, we consider the disclosure of this application to be enabling. The passages relied upon by the examiner from Mark '584 and copending Serial No. 06/876,819 are merely examples of work which is *outside* the claims on appeal. The record before us establishes that for a given protein having cysteine residues, one skilled in the art would be able to routinely determine whether deletion or replacement of the cysteine residues would result in a mutin which is within the claims on appeal.

To the extent that the examiner is concerned that undue experimentation would be required to determine other proteins suitable for use in the present invention, we find Ms. Wang's declaration to be persuasive that only routine experimentation would be needed for one skilled in the art to practice the claimed invention for a given protein. The fact that a given protein may not be amenable for use in the present invention in that the cysteine residues are needed for the biological activity of the protein does not militate against a conclusion of enablement. One skilled in the art is clearly enabled to perform such work as needed to determine whether the cysteine residues of a given protein are needed for retention of biological activity.

The examiner's rejection under 35 USC §112 first paragraph, is reversed.

### NEW GROUND OF REJECTION

Claims 1 through 5, 45 through 53, 55 and '57 through 67 are rejected under 35 USC §102(e) as being anticipated by Mark '584 or Mark '585.

The present application lists four co-inventors, Mark, Lin, Lu and Wang. Appellants state on page 1 of the present specification that this application has two lines of parent applications. It is the first line of parent applications, i.e., Serial Nos. 06/564,224, 06/486,162 and 06/435,154 which is of present interest.

This application is stated to be a continuation-in-part of Serial No. 06/564,224 which is a continuation-in-part of Serial No. 06/486,162 which is a continuation-in-part of Serial No. 06/435,154. Each of these parent applications lists only three inventors, Mark, Lin and Lu. Wang, who is a co-inventor of the present application, is not a co-inventor in the parent applications. Mark '584 issued from Serial No. 06/564,224. Mark '585 is a division of Serial No. 06/564,224, and shares common parentage with Mark '584 of Serial Nos. 06/486,162 and 06/435,154.

In order for the present claims to be entitled under 35 USC §120 to the benefit of the earlier filing date of any of the parent applications, their subject matter must be disclosed in the parent applications in the manner provided by 35 USC §112, first paragraph, including the description requirement of this section of the statute. *In re van Langenhoven*, 458 F.2d 132, 173 USPQ 426 (CCPA 1972).

[2] Here, our review leads us to the conclusion that the earliest filing date the present generic claims are entitled to is the December 20, 1983 filing date of parent application Serial No. 06/564,224 since this appears to be the first application in this chain which sets forth a generic description of the synthetic mutants of the present invention. Parent application Serial No. 06/486,162 describes only a synthetic mutin of IFN- $\beta$ . The entire original disclosure of Serial No. 06/486,162 describes and is strictly limited to synthetic mutants of IFN- $\beta$  except for original claim 20 of that application which was directed to "a nucleotide primer for mutagenesis, comprising an oligonucleotide of about 12 to about 24 bases." The specification of this application contains a corresponding disclosure of this generic nucleotide primer. However, comparing this generic disclosure of a nucleotide primer with that of the present application, i.e., claim 64, it is apparent that claim 20 of this parent application does not provide descriptive support for the *broader* oligonucleotide disclosed and claimed in this application. Thus, none of the present claims are entitled to the benefit of the earlier filing date of Serial No. 06/486,162, at best, only Serial No. 06/564,224.

Having made this determination, we find that Mark '584 or Mark '585 is available as prior art against the appealed claims under 35 USC §102(e) as these patents are by "others" having the effective filing date required by this section of the statute. The effective filing date of these two references, to the extent they disclose synthetic mutants of IFN- $\beta$ , is October 19, 1982, the filing date of common parent application Serial No. 06/435,154. They are anticipatory of the claims included in this rejection in that these references describe the IFN- $\beta$  synthetic mutin species of the present generic claims. *In re May*, 574 F.2d 1082, 1089, 197 USPQ 601, 607 (CCPA 1978).

Claims 68 and 69 are rejected under 35 USC §103 as being unpatentable over Mark '584 or Mark '585.

These claims are directed to a therapeutic formulation which comprises an effective amount of the mutin of the present inven-



tion and at least one other anti-cancer or anti-viral compound, e.g., gamma-interferon. While Mark '584 and Mark '585 describe such a therapeutic formulation, this description does not appear in common parlance. Serial No. 06/486,162. This parent application only indicates that the synthetic mutin of IFN- $\beta$  is useful for treatment of viral infections, and various types of cancer where interferon therapy is indicated.

However, in view of the disclosed utility of the synthetic mutin of IFN- $\beta$  as an anti-cancer or anti-viral compound in Serial No. 06/486,162, it would have been *prima facie* obvious to one of ordinary skill in the art to use the disclosed synthetic mutins of IFN- $\beta$  in combination with other known anti-cancer or anti-viral compounds such as gamma-interferon. *In re Kerkhoven*, 626 F.2d 846, 205 USPQ 1069 (CCPA 1980).

Any request for reconsideration or modification of this decision by the Board of Patent Appeals and Interferences based upon the same record must be filed within one month from the date of the decision (37 CFR 1.197). Should appellants elect to have further prosecution before the examiner in response to the new rejection under 37 CFR 1.196(b) by way of amendment or showing of facts, or both, not previously of record, a shortened statutory period for making such response is hereby set to expire two months from the date of this decision.

37 CFR 1.136(a) does not apply to the times for taking any subsequent action in connection with this appeal.

REVERSED

37 CFR 1.196(b)

## Court of Appeals, Federal Circuit

In re Iwahashi

No. 89-1019

Decided November 7, 1989

## PATENTS

### 1. Patent grant — Inventions patentable (§105.05)

Assertion that claim includes or is directed to algorithm does not automatically require finding that claim is directed to non-statutory subject matter, since 35 USC 101 expressly includes processes as category of inventions which may be patented, and since every step-by-step process, whether it is electronic, chemical, or mechanical, involves algorithm in broad sense of term.

### 2. Patent grant — Inventions patentable (§105.05)

Fact that apparatus, in claim for auto-correlation circuit for use in pattern recognition, operates according to mathematical algorithm does not itself render claim non-statutory under 35 USC 101; rather, showing, when claim is analyzed as whole, that algorithm is implemented in specific manner to define structural relationships between claim's physical elements warrants finding that claim is directed to statutory subject matter.

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Application for patent, serial no. 454,022, filed Dec. 28, 1982, by Hiroyuki Iwahashi, Yoshiaki Nishioka, and Mitsuhiro Hakari-dani, for auto-correlation circuit for use in pattern recognition. From decision rejecting claim, applicants appeal. Reversed.

Charles Gorenstein, of Birch, Stewart, Kolasch & Birch (Michael K. Mutter, with him on brief), Falls Church, Va., for appellant.

John C. Martin, associate solicitor (Fred E. McKelvey, solicitor, with him on brief), for appellee.

Before Nichols, senior circuit judge,\* and Rich and Bissell, circuit judges.

Rich, J.

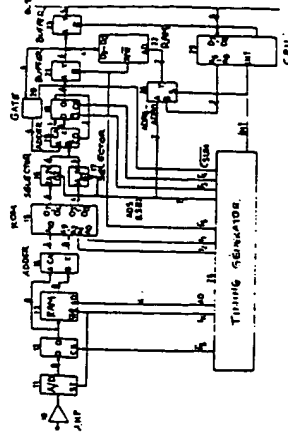
This appeal is from the decision of the United States Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (board), dated May 24, 1988, adhered to on reconsideration, affirming the examiner's final rejection of the single claim of applicants' patent application serial No. 454,022, filed December 28, 1982, entitled "Auto-Correlation Circuit for Use in Pattern Recognition." The sole ground of rejection is that the subject matter claimed is nonstatutory under 35 U.S.C. §101 because it is merely a mathematical algorithm. We reverse.

The real party in interest, according to appellants' brief, is Sharp Kabishiki Kaisha (Sharp Corporation).

\* Judge Nichols heard oral argument but, due to illness, did not participate in the decision.

The opening sentence of the specification states: "This invention relates to an auto-correlation unit for use in pattern recognition to obtain auto-correlation coefficients as for stored signal samples." The embodiment more particularly discussed as a species of pattern recognition is voice recognition. The prior art calculation of auto-correlation coefficients is described as being based on a calculation formula involving a multiplication step. The specification states the disadvantage to be as follows:

FIG. 2



We shall not attempt a description of the electronic circuitry shown by these drawings beyond explaining, for the better understanding of the claim, that the units designated "ROM" and "RAM" are, respectively, a read only memory and a random access memory, terms well understood by those skilled in the art. "CPU" is a central processing unit. In this case, the function of the ROM, 2 or 15, which is a permanent information storage device, is to deliver as output the square of a number fed to it as input. It is the electronic equivalent of a table in which one can look up the square of numbers over a desired range.

We next reproduce the claim on appeal and do so by presenting a copy of the claim as it has been presented in the Solicitor's brief, to which we have added the letters in brackets designating at [a] the preamble or introductory clause and at [b] through [h] the several means-plus-function and other elements of the combination of elements recited. Under the heading "Drawings" (the Solicitor's heading was "Fig. 1"), we have copied verbatim the Solicitor's designations. Element [d], it will be noted, is not in means-plus-function form but specifies a "read only memory" or ROM, as the Solicitor says. Element [f] is an anomalous clause seemingly intended to indicate what data are stored in the ROM. It is not clear that a means for storing anything in the ROM is part of the disclosed "unit" since the application indicates that the squared values are "previously memorized" in the ROM. However, that has nothing to do with the sole question before us which is whether the claim as a whole is, in the words of the Solicitor, "directed to non-statutory subject matter," so we shall not comment further on element [f].

Those state-of-the-art units for calculation of the auto-correlation coefficients have the disadvantage of requiring expensive multipliers and also complicated circuitry. As a result the auto-correlation unit circuitry within the entire pattern recognition apparatus is proportionately large and auto-correlation calculation demands a greater amount of time during recognition.

The principal object of this invention is to provide an auto-correlation unit for pattern recognition which evaluates auto-correlation coefficients by means of a simple circuitry without the need for an expensive multiplier as well as eliminating the above discussed disadvantages. Underlying the auto-correlation unit claimed, is a plethora of mathematical demonstration by which the applicants purport to show that the approximated value of the desired coefficient can be obtained without multipliers by obtaining the square of the sum of two of the factors in the equation and calculating the auto-correlation coefficient therefrom according to a stated formula. The specification concludes:

As explained in the foregoing, this invention offers a highly cost effective auto-correlation unit for pattern recognition with simple circuitry without the need to use an expensive multiplier, but which has comparatively high accuracy and can, moreover, calculate auto-correlation coefficients at high speed.

Fig. 1 of the application drawings is described as "a block diagram schematically showing an embodiment of this invention" and appears as follows:

